University of Alberta

The Role of ABCA1 in tyrHDL-mediated Enhancement of Cholesterol Mobilization

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Department of Biochemistry

Edmonton, Alberta
Fall 2006

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Your file Votre référence
ISBN: 978-0-494-22334-5
Our file Notre référence
ISBN: 978-0-494-22334-5

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Abstract

Tyrosyl radical-oxidized high density lipoprotein (tyrHDL) has an enhanced ability to stimulate the mobilization of cellular cholesterol for efflux for the formation of HDL particles, and to reduce atherosclerosis in an animal model. ATP-binding cassette transporter A1 (ABCA1) promotes the release of excess cellular cholesterol in the process of forming HDL particles. We postulated that tyrHDL enhances the mobilization of cellular lipids for HDL formation by enhancing the expression or activity of ABCA1.

TyrHDL requires the presence of functional ABCA1 to enhance mobilization of cholesterol from cells. Furthermore, tyrHDL increases ABCA1 protein in human aortic smooth muscle cells and human skin fibroblasts, suggesting a potential role in the stabilization of ABCA1 from proteolysis. As well, tyrHDL directly cross-links to ABCA1, and its activity requires the actions of the liver X receptor. Hence, tyrHDL has therapeutic implications for the prevention and treatment of atherosclerosis.

Acknowledgement

I would like to thank my supervisor Dr. Gordon Francis for accepting me into graduate studies and for his guidance throughout the years. You have given me a valuable life experience that will serve me well in my future journeys.

I would like to thank my supervisory committee members Dr. Dennis Vance and Dr. Michael Schultz as well as my external examinee Dr. Richard Lehner for their support throughout my graduate program.

Thank you to Dr. David Mangelsdorf, University of Texas Southwestern, for providing the LXR double knockout mouse tissues.

Much thanks must be given to Teddy Chan for outstanding technical support, for training me in the lab, and for introducing me to the wonderful world of golf! I would also like to thank Dr. Hong Choi, Dr. Emma Waddington, Diana Lee, and Dr. Emmanuel Boadu for their helpful comments, conversations, and insights.

Thank you to the MCBL group, and to the members of the MCBL group for your support and friendship.

I dedicate this to my Mother, Maly Korn

Who was always there at key moments in my life, who never stopped believing in me, and who always pointed me in the right direction. Thank you for giving me a future, for your support and guidance, and most importantly, for your strength. I would also like to dedicate this to my family. We have come far from where we were, but sometimes it is the journey and not the destination that shapes who we are. I am glad to have taken this journey with you all.

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List of Abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCA1-KO	ATP-binding cassette transporter A1 knockout
ABCG1	ATP-binding cassette transporter G1
ACAT	acyl-CoA:cholesterol acyltransferase
ApoA-I	apolipoprotein A-I
ApoA-II	apolipoprotein A-II
ApoB	apolipoprotein B
ApoE	apolipoprotein E
apoE-KO	apolipoprotein E knock out
BSA	essentially fatty acid-free bovine serum albumin
CAD	coronary artery disease
cDNA	complementary deoxyribonucleic acid
CE	cholesteryl esters
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
DMEM	Dulbecco's modified Eagle's medium
DMEM/BSA	DMEM containing 1 mg/ml BSA
dNTP	deoxyribonucleotide triphosphate
DSP	dithiobis(succinimidyl propionate)
DTPA	diethylenetriamine pentaacetic acid
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FC	free cholesterol
h	hours
HASMC	human aortic smooth muscle cells
HDL	high density lipoproteins
HDL-C	high density lipoprotein cholesterol
HRP	horseradish peroxidase
HSF	human skin fibroblasts

IDL	intermediate density lipoproteins
JAK 2	Janus kinase 2
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoproteins
LDL-C	low density lipoprotein cholesterol
LDLr	low density lipoprotein receptor
LDLr-KO	low density lipoprotein receptor knockout
LPL	lipoprotein lipase
LXR	liver X receptor
min	minutes
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MSF	mouse skin fibroblasts
NO	nitric oxide
PBS	phosphate-buffered saline
PBS/BSA	PBS containing 1 mg/ml BSA
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PEST	sequence of ABCA1 rich in proline, glutamate, serine, threonine
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipids
PLTP	phospholipid transfer protein
PM	plasma membrane
PS	phosphatidylserine
RIPA	radio-immunoprecipitation assay
RCT	reverse cholesterol transport
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SR-BI	scavenger receptor class B, type I
TAG	triacylglycerols

TBS	Tris-buffered saline
TBS/T	TBS containing 0.1% Tween 20
tyrHDL	tyrosyl radical-oxidized HDL or tyrosylated HDL
VLDL	very low density lipoproteins

CHAPTER 1: INTRODUCTION

Diseases related to atherosclerosis such as heart attack and stroke are the leading cause of death worldwide. Increased plasma levels of the high density lipoproteins (HDL) are protective against the development of atherosclerosis. Hence, novel methods to increase HDL formation for the treatment and prevention of atherosclerosis would greatly benefit all at large. The main anti-atherogenic property of HDL is believed to be its involvement in the transport of excess cholesterol from tissues including the artery wall to the liver to be excreted into bile. It was shown that the protein ATP-binding cassette transporter A1 (ABCA1), found on the surface of most cells, mediates active transport of phospholipids and cholesterol out of cells upon interaction with the major protein of HDL, apolipoprotein A-I (apoA-I). This process generates new HDL particles. Modification of HDL by tyrosyl radical (tyrosylated HDL or tyrHDL) has also been shown to markedly increase the ability of HDL particles to remove cholesterol from cells and increase HDL formation when compared to native HDL particles. This effect is absent in cells lacking functional ABCA1, indicating at least the requirement of ABCA1 for the action of tyrHDL. We have demonstrated that tyrHDL, like apoA-I, increases the level of ABCA1 protein in cells. The activity of tyrHDL seems to depend on the liver X receptor pathway, a transcription factor responsible for the transcription of genes involved in cholesterol homeostasis. Also like apoA-I, but unlike HDL, tyrHDL is capable of being cross-linked to ABCA1, suggesting a direct tyrHDL-ABCA1 interaction. Additional studies using protein fragments of tyrHDL suggest, however, that a direct binding interaction between tyrHDL and ABCA1 may not be necessary for tyrHDL to enhance the activity of ABCA1 in cells. The ability of tyrHDL to enhance the activity of ABCA1 demonstrated by these studies may suggest important new ways to increase the formation of HDL for the prevention and treatment of atherosclerosis.

1.1 Introduction to cardiovascular disease

1.1.1 Global burden of atherosclerosis and cardiovascular disease

Coronary artery disease (CAD) as a consequence of atherosclerosis (hardening of the arteries) is the leading cause of death in the developed and developing countries (*1*). It was estimated that in 1996, 29% of the world's deaths could be attributed to cardiovascular disease and that half of these deaths were due to atherosclerosis of the

coronary arteries (*2*). Major risk factors for the development of CAD include age, dyslipidemia, diabetes mellitus, smoking, hypertension, and genetic factors that predispose to some of these other major predictors. With respect to dyslipidemia, much attention is given to the plasma levels of low density lipoprotein (LDL) and HDL particles. A high plasma level of LDL cholesterol (LDL-C) is proatherogenic, whereas individuals with high HDL cholesterol (HDL-C) are protected against atherosclerosis. The very strong relationship between elevated HDL-C levels and protection against atherosclerotic vascular disease suggests an increased understanding of HDL formation and metabolism is critical to our ability to reduce lives lost or impaired as well as the treatment costs incurred by atherosclerosis.

1.2 Atherogenesis

1.2.1 Structure of the artery wall

The artery wall is made up of three distinct layers (**Figure 1.1**) (3). The intimal layer lies just beneath the endothelial cells that line the artery wall. Under normal circumstances, the intima is acellular and contains primarily connective tissue including the internal elastic lamina. Beneath the intima is the medial layer. The medial layer consists of smooth muscle cells as the primary cell type, collagen, and fibroelastic connective tissue. Smooth muscle cells are responsible for regulating the size of the lumen of the artery wall upon contraction or relaxation. The outermost layer of the artery wall is termed the adventitia, the artery wall coat consisting of dense fibroelastic tissue that merges with nearby connective tissue.

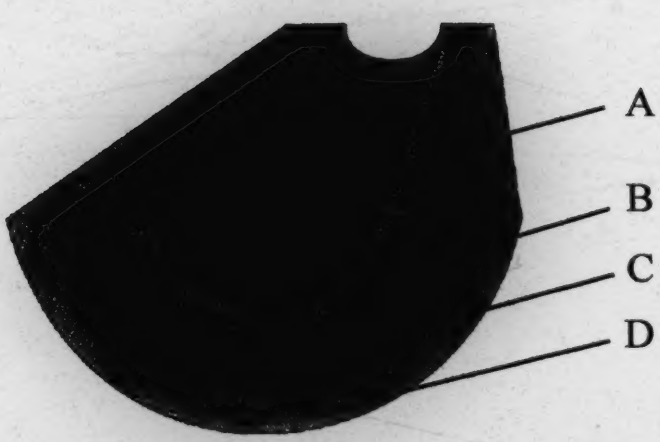

Figure 1.1: Layers of the artery wall. The endothelium (A) is in contact with the blood and overlies the intima (B). Beneath this is the media (C), consisting of layers of smooth muscle cells. Underneath the media is the adventitia (D). The figure was adapted from Ross, R. by permission from Macmillan Publishers Ltd: [Nature] (3), copyright (1993) with permission.

1.2.2 The atherosclerotic lesion: formation and progression

It is hypothesized that atherosclerosis is an inflammatory disease initiated by endothelial injury or dysfunction (4). Endothelial injury can occur from a variety of causes, including elevated cholesterol levels, elevated blood sugar in diabetes, high blood pressure, free radicals and other toxins in cigarette smoke, elevated homocysteine levels, and possibly infectious causes including herpesviruses or *Chlamydia pneumoniae* (5). If injury to the endothelium occurs unabated, this process will result in an advanced, complicated atherosclerotic lesion. The endothelium responds to injury by increasing its expression of cell adhesion molecules and permeability to surrounding platelets and leukocytes, and by releasing vasoactive molecules, cytokines, and growth factors that attract nearby inflammatory cells. These cells, primarily monocytes and some T-lymphocytes and neutrophils, then migrate through the endothelium into the intimal space. The inflammatory reaction also stimulates migration and proliferation of smooth muscle cells from the media to the intimal layer. Monocytes and macrophages in the intimal space release more pro-inflammatory cytokines and chemokines, exacerbating the

inflammatory response and lesion formation. As a result, massive amounts of monocytes and T-lymphocytes migrate from the blood and multiply within the lesion. Activation of these cells leads to release of hydrolytic enzymes and chemotactic molecules that induce further damage and attract more proinflammatory cells, creating a cycle of inflammation and lesion infiltration (3, 6).

Increased LDL-C is associated with atherosclerosis. The increased permeability of the damaged endothelium allows LDL to easily cross into the intimal layer. In addition, LDL-C present in high levels in plasma might also traverse the endothelial layer and accumulate in the artery wall even in the absence of significant endothelial damage. LDL can become trapped in the intimal layer due to binding of positively charged lysine and arginine residues on apoprotein B100, the major protein of LDL, by negatively charged proteoglycans in the intimal space. When LDL particles become trapped, they undergo progressive oxidation and/or aggregation, followed by internalization by surrounding macrophages and smooth muscle cells expressing scavenger receptors. This forces the macrophages and smooth muscle cells to esterify the excess LDL-derived cholesterol, facilitating the massive accumulation of cholesteryl esters (CE) resulting in the formation of fatty streaks and foam cells (5). This process of sequestration and removal of oxidized LDL is initially a protective mechanism against lesion formation; however, once CE accumulate beyond the macrophage's ability to metabolize the cholesterol, the atheroma enlarges and protrudes into the lumen of the artery, altering blood flow. CE-rich plaques are unstable, and even lesions occluding as little as 20% of the artery lumen can rupture, leading to thrombus formation on the ruptured plaque and potential complete sudden occlusion of the artery resulting in a myocardial infarction (heart attack) or stroke.

1.3 Lipoprotein structure and function

1.3.1 Introduction to lipoproteins

Cholesterol and triacylglycerols (TAG) are water insoluble lipids that are solubilized for transport in plasma by way of lipoprotein particles. These lipids are packaged into complexes with water-soluble proteins known as apoproteins (also known as apolipoproteins or apos). Delivery and secretion of cholesterol and TAG require a

combination of synthesis, catabolism, and remodeling between lipoprotein particles, enzymes, and cellular proteins. There are unique classes of lipoprotein particles; however, all of them have common structural features (**Figure 1.2**). Lipoprotein particles consist of a neutral lipid core rich in CE and/or TAG, with a monolayer of phospholipids (PL), free (unesterified) cholesterol (FC), and apoproteins surrounding the neutral lipid core. The amphipathic nature of the apoproteins allows their polar surface to be exposed to the aqueous plasma whereas the hydrophobic surface faces the inner core. In addition, most apoproteins are exchangeable in the sense they are able to dissociate from the lipoprotein surface, relative to their degree of lipid affinity, to be free in plasma or to associate with other lipoproteins. The exception is the very large and hydrophobic apoprotein B (apoB), which remains bound to its lipoprotein surface.

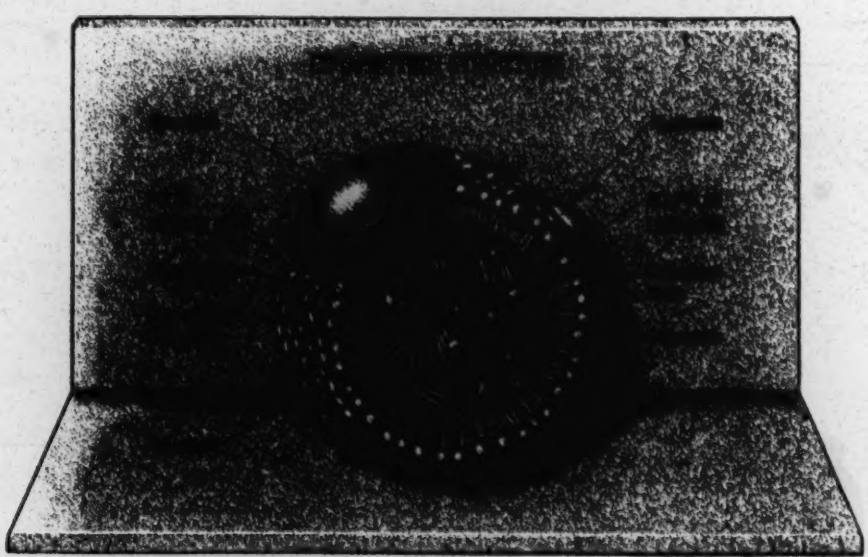

Figure 1.2: Structure of a typical lipoprotein particle. The outer monolayer consists of amphipathic phospholipids and free cholesterol. The hydrophobic core can be composed of TAG and/or CE. Apoproteins embedded in the outer coat help to solubilize the particle in the plasma. The figure was obtained from Grundy, S.M. (7).

Lipoproteins are classified broadly based on their density. The largest and least dense are chylomicrons, which have the highest concentration of TAG, and contain apos A-I, B48, C, and E in the shell of the particle. Chylomicrons are synthesized and secreted

by the small intestine after dietary lipids and intestinal cholesterol are digested, absorbed and repackaged by enterocytes. The next largest lipoproteins are very low density lipoproteins (VLDL), which are also rich in TAG and contain a single molecule of apoB100 as their major structural apoprotein. VLDL are synthesized and secreted by the liver, and circulate within the bloodstream where their TAG are digested by lipoprotein lipase (LPL), situated in highest concentrations in endothelial cells of capillaries overlying adipose tissue, and cardiac and skeletal muscle. Digestion of VLDL TAG provides these tissues with necessary fatty acids for storage or immediate sources of energy. The products of VLDL catabolism are the intermediate density lipoproteins (IDL), also known as VLDL remnants, followed by LDL. LDL are the most cholesterol-rich particles in the bloodstream. Delivery of cholesterol to peripheral (non-hepatic) tissues is mediated mainly by LDL particles, through uptake by the LDL receptor (LDLr). The final and most dense class of lipoprotein particles are the HDL. The density of HDL particles range from 1.063 to 1.21 g/mL, contain mostly CE in their core, and have apos A-I and A-II as their primary structural apoproteins. HDL play a major role in the transport of excess cholesterol from the periphery to the liver (8).

1.3.2 Structure and biochemical composition of HDL

ApoA-I is the major apoprotein of HDL (1-4 copies per particle), making up ~70 percent of the total protein content on HDL (9). ApoA-I is a 243 amino acid amphipathic protein, containing 10 α-helical domains, produced and secreted by the intestine and the liver (10). ApoA-II is a 77 amino acid protein, also amphipathic and containing 3 α-helical domains, produced and secreted by the liver (11, 12). In addition to apoA-I and A-II, HDL particles may also contain other exchangeable apoproteins including apoA-IV, A-V, C-I, C-II, C-III, D, J, L and E as secondary apoproteins (13). HDL also carry plasma proteins involved in modulation of HDL lipid content, including lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP) (8). ApoA-I exists in three forms in plasma: as a lipid-poor protein, in discoidal HDL, and in spherical HDL (14). On agarose gel electrophoresis, which separates lipoproteins based on particle charge, α-migrating HDL particles are typically spherical and contain two or more molecules of apoA-I plus PL,

FC, CE and TAG and account for the major proportion of HDL in plasma, which include HDL_2 (1.063<density<1.125 g/mL) and HDL_3 (1.125<density<1.21 g/mL). Pre-β migrating HDL particles are either lipid-poor apoA-I or discoidal particles consisting of two or three molecules of apoA-I complexed with a bilayer of PL and possibly small amounts of FC (8).

1.3.3 Intravascular remodeling of HDL

HDL is remodeled within the plasma compartment by a complex interplay with plasma enzymes and proteins responsible for the esterification of cholesterol on HDL, and the transfer or exchange of different lipid species between lipoprotein particles. The major apoprotein of HDL, apoA-I, is primarily synthesized in the liver. Liver apoA-I is secreted as lipid-free/poor apoA-I or as discoidal nascent HDL particles made up of poorly lipidated apoA-I that has received some lipids upon the initial interaction with liver ABCA1. These nascent HDL particles then travel to the periphery to pick up excess cholesterol from peripheral tissue (expanded on in detail in *section 1.4.3*). LCAT plays an important role in the maturation of nascent HDL particles as it catalyzes the esterification of FC on the surface of HDL, with CE moving to the core of the particle to generate spherical HDL particles. The action of LCAT maintains a low concentration of FC on the HDL particle surface, allowing additional peripheral cell surface cholesterol to flow down a concentration gradient to the HDL surface (15). The activity of LCAT is crucial for HDL maturation; genetic LCAT deficiency syndromes are associated with markedly reduced HDL-C and apoA-I levels because smaller, discoidal HDL are cleared much more rapidly from the circulation (16). CETP promotes the transfer of CE from HDL in exchange for TAG from apoB-containing lipoproteins (LDL, IDL, VLDL, and chylomicrons) (17). TAG enrichment of HDL by CETP may improve the catabolism of HDL by hepatic lipase, which hydrolyzes HDL TAG and PL, generating smaller HDL_3 particles and free apoA-I that can then recirculate in further rounds of tissue cholesterol removal (18). CETP-deficient patients have markedly elevated levels of HDL-C (19) and reduced turnover of apoA-I (20), with net effects on atherosclerosis reported to be either positive (21) or negative (22, 23). PLTP transfers surface PL from TAG-rich lipoproteins to HDL during TAG lipolysis by LPL (24). The net role of hepatic lipase in modulating

atherogenic risk remains controversial. It is thought that hepatic lipase activity is inversely correlated with the development of atherosclerosis (25), however some studies suggest that hepatic lipase expression is proatherogenic (26, 27).

1.3.4 Transport of cholesterol to peripheral tissues

Cholesterol is a vital component of cellular membranes and is the precursor for bioactive molecules such as steroid hormones, bile acids, oxysterols, and vitamin D. Cells can synthesize their own cholesterol endogenously, or acquire it via LDL uptake. LDL cholesterol is derived from VLDL secreted by the liver, or tissue cholesterol delivered to LDL from HDL by the actions of CETP. LDL is taken up by tissues via receptor-mediated endocytosis (**Figure 1.3**).

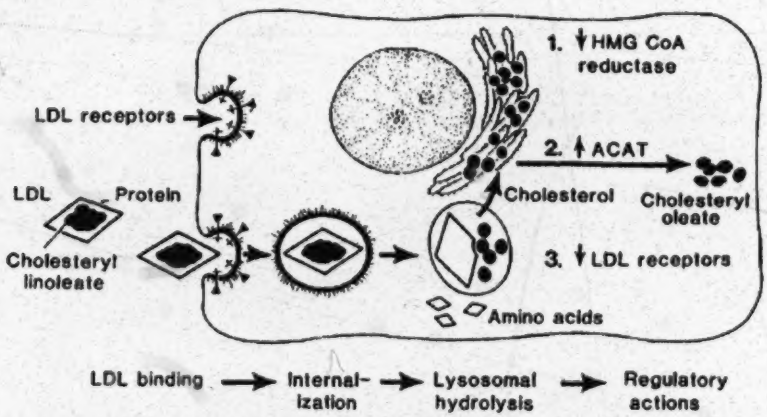

Figure 1.3: Receptor-mediated endocytosis of LDL. LDL particles bind to LDL receptors clustered in clathrin-coated pits. The receptor-LDL complex is internalized in coated vesicles. The LDL particles are delivered to lysosomes where apoB is digested and CE are hydrolyzed, releasing cholesterol for subsequent diverse intracellular actions. The figure was obtained from Brown, M.S., and Goldstein, J.L. (28) with permission.

In this pathway, apoB of LDL particles binds to the LDLr expressed on the surface of cells, and the whole particle is endocytosed and transported to lysosomes for degradation. Excess FC derived from LDL is converted in the endoplasmic reticulum to CE by acyl-CoA:cholesterol acyltransferase (ACAT) (**Figure 1.3**). ACAT thus converts FC into nontoxic cytoplasmic CE droplets. Increased cellular cholesterol also downregulates new cholesterol synthesis and further LDL uptake by cholesterol- and oxysterol-dependent

inhibition of HMG-CoA reductase and LDLr expression (**Figure 1.3**) (29). In this way, the regulation of cholesterol synthesis and influx is tightly controlled to prevent against cholesterol toxicity.

1.4 Physiological functions of HDL

1.4.1 Epidemiologic evidence for the protective action of HDL

Numerous observational and epidemiological trials have shown that high plasma HDL-C levels are protective against atherosclerosis. The recent INTERHEART study of the major predictors of first myocardial infarction worldwide identified the ratio of apoB to apoA-I as the strongest predictor of risk for coronary heart disease (30). This ratio can be interpreted as indicating the balance of influx of cholesterol to the artery wall from apoB-containing lipoproteins (LDL, VLDL and chylomicrons and their remnants), and removal of this cholesterol by apoA-I-containing particles (HDL). Earlier studies including the Framingham Heart Study, the Prospective Cardiovascular Munster study, the Helsinki Heart Study, the Veterans Affairs High Density Lipoprotein Trial, and the Multiple Risk Factor Intervention Trial had all found plasma HDL-C levels to be a stronger predictor of the development of CAD than LDL levels (31-33). In the Framingham Heart Study, patients free of any apparent cardiovascular disease (CVD) were enrolled and their lipid parameters measured. Those who had the highest HDL-C levels at study enrollment had the lowest risk of developing CAD during the next 35 years of study (34). The association of HDL-C with CVD was noted at all levels of total cholesterol or LDL-C. In addition, further analysis of the Framingham, the Multiple Risk Factor Intervention Trial, the Lipid Research Clinics Prevalence Mortality Follow-up Study, and the Coronary Primary Prevention Trial that adjusted for age, blood pressure, smoking, body mass index and LDL-C, showed a consistent and strong inverse relationship between HDL-C levels and CAD event rate (32).

One criticism of clinical studies involving the pharmacological manipulation of plasma HDL levels is that drugs used to raise HDL-C may also directly decrease LDL-C and lower TAG, thereby confounding the effects of raising HDL-C alone. The Veterans Affairs High Density Lipoprotein Trial using gemfibrozil represented a unique study in that it was the first clinical trial to suggest clinical benefits from pharmacologically

increasing HDL-C and lowering TAG levels without any reduction in LDL-C (31). This is in favor of the theory that there is a clear and consistent inverse relationship between HDL-C and CAD, and that HDL levels are a much stronger predictor of risk for CVD than LDL levels. These large.international trials suggested that each 1% (or 1 mg/dL) increase in HDL-C was linked to a 2 to 3% reduction in the development of CAD (32). Although the exact *in vivo* action of HDL for the protection against atherosclerosis is not well understood, these studies suggest raising HDL-C is an important goal for the treatment and prevention of CVD.

1.4.2 Cardioprotective actions of HDL

In vitro evidence suggests several potential cardioprotective actions of HDL. HDL may be protective by hydrolyzing oxidized lipids on LDL via paraoxonase (35), thereby neutralizing the atherogenic effects of oxidized LDL. Paraoxonase is an arylesterase that is transported on HDL particles and is believed to be responsible for cleaving oxidized fatty acids from PL of oxidized LDL, rendering it less atherogenic (35). ApoA-I, the major protein of HDL, may also have antioxidant activity by removing lipid hydroperoxides on LDL and HDL (35, 36). HDL has also been shown to have anti-inflammatory properties by inhibiting the expression of the endothelial cell adhesion molecules VCAM-1 and ICAM-1, in addition to chemotactic cytokines such as monocyte chemotactic protein-1 (36), thereby inhibiting monocyte recruitment, binding, and migration into the intima that occurs in the early stages of lesion formation. Endothelial dysfunction is characterized by decreased bioavailability of nitric oxide (NO), a potent vasodilator. HDL is also thought to modulate endothelial function by stimulating NO production and inhibiting thrombin-induced platelet aggregation, thereby inducing vasodilation and reducing the risk of thrombosis, respectively (8).

1.4.3 Reverse cholesterol transport and HDL formation

The primary and most widely accepted role of HDL in protecting against atherosclerosis is its ability to promote reverse cholesterol transport (RCT). The RCT pathway was initially proposed by Glomset in 1968 (37). The pathway describes the delivery of cholesterol, which in most cell types cannot be catabolized, from peripheral

tissues such as macrophages, foam cells, and atherosclerotic plaques to the liver to be excreted from the body in bile (**Figure 1.4**). ApoA-I secreted by the liver and intestine as lipid-free/poor apoA-I or nascent discoidal (pre-β-migrating) HDL that picked up some lipids by way of liver ABCA1, travel to the periphery and interact in the interstitial (sub-endothelial) space with the cell surface membrane protein ABCA1 of systemic cells to receive FC and PL. The newly acquired cholesterol is then esterified by LCAT to transform the discoidal HDL into an α-migrating spherical HDL particle.

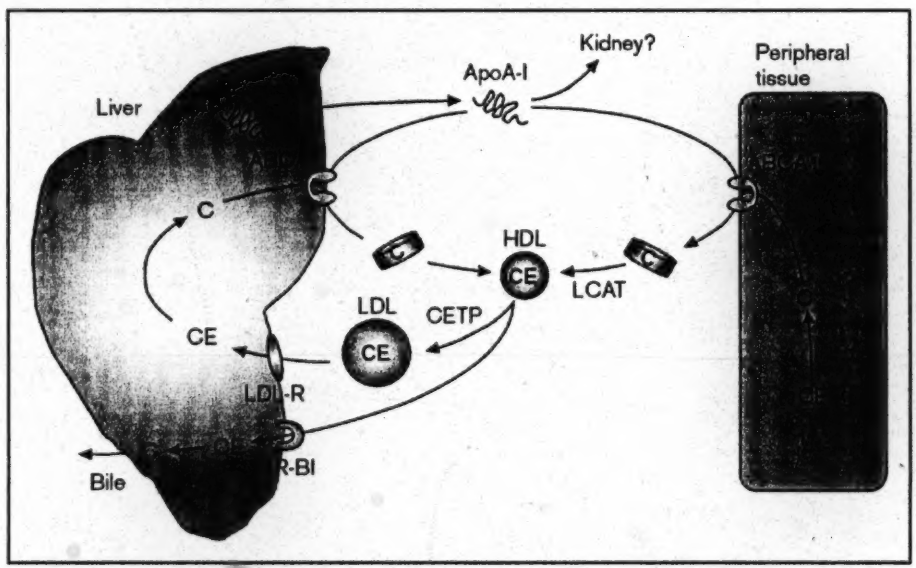

Figure 1.4: A simplified model of reverse cholesterol transport. The liver secretes lipid-free/poor apoA-I and partially-lipidated apoA-I (discoidal HDL) via liver ABCA1 into the circulation. Peripheral cells such as macrophages in the arterial wall release cholesterol and PL to the cholesterol-poor apoA-I particles via ABCA1, and thus convert them into cholesterol-rich HDL particles. After a series of reactions with LCAT and CETP in the plasma, HDL-cholesterol is delivered back to the liver via the scavenger receptor class B, type I (SR-BI), leaving lipid-poor apoA-I for further cycling in the reverse cholesterol transport pathway. The figure was obtained from Oram, J.F. (38) with permission.

Further remodeling of the HDL particle to progressively larger and more mature spherical HDL complexes occurs by HDL receiving additional cellular cholesterol by non-ABCA1-dependent mechanisms, and by interactions with PLTP and CETP. The cholesterol that is transferred from HDL to apoB-containing lipoproteins by way of

CETP can be transferred back to the liver for recycling via the LDLr or the LDL receptor-related protein, or may once again be taken up by systemic tissues. Delivery of HDL-C to the liver is predominantly mediated by the scavenger receptor class B, type I (SR-BI). Liver SR-BI facilitates the unidirectional selective uptake of HDL CE (39). These CE are thought to be preferentially metabolized into bile acids and eliminated through the gastrointestinal tract, thus completing the RCT pathway (17, 40, 41). This was demonstrated when administration of HDL containing radiolabeled cholesterol to a patient resulted in the preferential shunting of those labeled cholesterol into bile (42).

1.4.4 Protection of HDL against atherosclerosis and potential methods to raise HDL clinically

Prospective epidemiological studies support the hypothesis that high HDL-C levels limit atherosclerosis development. Similarly, *in vivo* animal studies have provided some of the best evidence for the atheroprotective actions of HDL. Early studies with transgenic mice overexpressing the human apoA-I gene and fed an atherogenic diet yielded increased plasma concentrations of apoA-I and HDL-C, and significant protection of these mice from the development of atherosclerosis (43). Further evidence to support the role of HDL in reducing atherosclerosis was apparent when transgenic mice overexpressing the human apoA-I gene were crossed with apoE-deficient mice, a strain that is highly susceptible to spontaneous atherosclerosis. In this animal model, the levels of HDL-C increased two-fold, which resulted in a significant decrease in development of atherosclerosis (44, 45). Liver-specific expression of human apoA-I in LDLr-deficient mice, another mouse model of atherosclerosis, also reduced the progression of atherosclerosis in mice fed a high cholesterol diet (46). Transgenic apoA-I has also been found to protect against atherosclerosis in rabbits, an animal model mimicking human lipoprotein metabolism more closely than mice. Human apoA-I transgenic rabbits fed a cholesterol-rich diet had significantly higher HDL-C levels and 50% less aortic lesion area and aortic lipid accumulation than the control group despite having similar levels of apoB-containing lipoproteins (47). The effectiveness of HDL in inhibiting atherogenesis was further characterized by injection of cholesterol-fed rabbits with either rabbit apoA-I or HDL plasma fractions. The injections induced marked

regression and inhibited the progression of aortic fatty streaks accompanied by decreased lipid deposition in the arterial wall (48-50). Perhaps the most compelling evidence for the protective role of HDL was presented when clinical trials showed that weekly intravenous injections of an apoA-I variant (apoA-I Milano) complexed with PL into human subjects with established CVD dramatically reduced atheroma volume by 4.2% after only five weekly treatments that only raised HDL levels acutely (51). Surprisingly, orally administered apoA-I mimetic peptides containing 18 D-amino acids (D-4F), which are resistant to enzymatic or acid hydrolysis in the digestive tract, markedly reduced atherosclerosis in mouse models without altering plasma cholesterol levels (52). Further studies with peptides as short as 4 amino acids in length (lysine-arginine-glutamate-serine, KRES) but no amphipathic alpha-helical structure have also been shown to reduce atherosclerosis in apoE-deficient mice (53). These studies increase the potential for peptides mimicking the actions of apoA-I to be developed as novel therapies for the treatment and prevention of atherosclerosis.

Clearly, methods to raise HDL clinically are an attractive target for the prevention of CAD. Currently, strategies to raise HDL include lifestyle modifications such as exercise, smoking cessation, weight control, moderate alcohol intake, and control of diabetes (54). Pharmacologic agents that increase HDL-C include niacin, which inhibits hepatic uptake of apoA-I and increases plasma pre-β HDL levels, as well as fibric acid derivatives, which are peroxisome proliferator-activated receptor α agonists that increase expression of apoA-I by the liver (54). The discovery that CETP-deficient patients had markedly raised levels of HDL-C and reduced LDL-C levels (19) has led to the development of drugs that inhibit CETP (55). Perhaps the most suitable candidate as a target for increasing HDL formation is the initial mediator of lipidation of apoA-I and other HDL apoproteins, ABCA1. ABCA1-mediated cholesterol efflux to apoA-I is now considered to be the rate-limiting step in the RCT pathway, providing a novel target to increase HDL particle formation therapeutically.

1.5 ABCA1-dependent lipid efflux in reverse cholesterol transport

1.5.1 Introduction to ABCA1

Investigations of a rare genetic cause of extremely low HDL levels, Tangier disease, have provided extremely valuable insights into the rate-limiting step of HDL particle formation, and a key predictor of plasma HDL-C levels. Tangier disease is a disorder characterized by low levels of plasma HDL and apoA-I, resulting in the massive accumulation of CE within various tissues including macrophages (56). Early studies in Tangier disease subjects indicated a deficiency of HDL apoproteins largely from a rapid catabolism of apoA-I and apoA-II, rather than a defect in the biosynthesis of those apoproteins (57). Further studies indicated PL (58) and cholesterol (58, 59) efflux to apoA-I and other HDL apoproteins (60) was impaired from Tangier disease cells. These studies suggested efflux of cellular lipids to lipid-poor apoA-I is a critical step in HDL particle formation, and a major determinant of circulating HDL-C levels. The defect in Tangier disease was subsequently identified as mutations in ABCA1 (61-65). ABCA1 is a member of the large superfamily of ABC membrane transporters (66), and is present on the surface of most cells to stimulate the delivery of cell cholesterol and PL to apoA-I and other HDL apoproteins including apoA-II and apoE (38). The critical importance of functional ABCA1 in determining plasma HDL-C levels is suggested by the near-absence of HDL particles in individuals with mutations in both ABCA1 alleles (Tangier disease), and approximately ½ normal plasma HDL-C levels in individuals with one functional ABCA1 allele (67, 68). It therefore appears that subsequent steps in the RCT pathway cannot compensate for an initial decrease in ABCA1 activity to normalize HDL-C levels. Tangier disease homozygotes and heterozygotes are consequently at increased risk of developing atherosclerosis (69, 70).

ABCA1 is a 2261-amino acid protein that utilizes ATP energy to transport lipids across membranes (66). It is a full ABC transporter, in that it contains two transmembrane domains and two ATP binding domains. Its structure is predicted to have 2 large extracellular loops that are highly glycosylated and linked by one or more cysteine bonds (**Figure 1.5**) (66, 71).

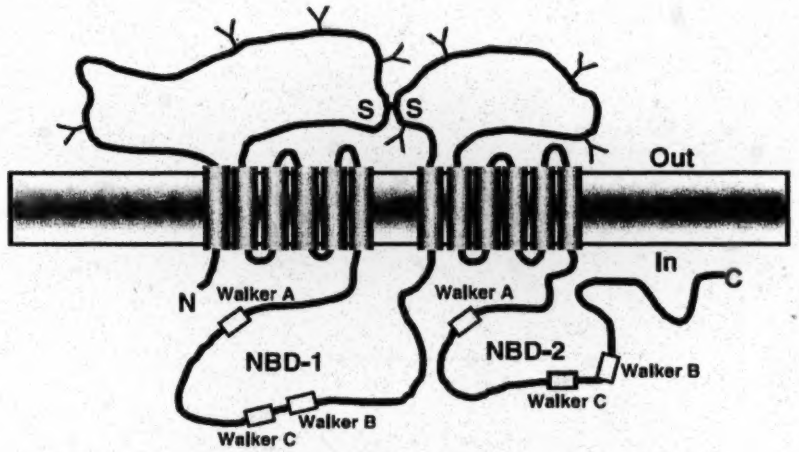

Figure 1.5: Current topological model of ABCA1. "Y" in the extracellular loops indicates approximate glycosylation sites, and S-S indicates 1 predicted disulfide bond. Nucleotide-binding domain 1 (NBD-1) and NBD-2 contain the highly conserved Walker A and Walker B sequences. The figure was obtained from Oram J.F. and Heinecke J.W. (72) and used with permission.

1.5.2 Role of ABCA1 in apoA-I-mediated cholesterol efflux

The active interaction of HDL proteins, particularly apoA-I, with cells to remove cellular lipids is the critical step in the initial production of HDL, without which no HDL is available for further passive removal of cell cholesterol or subsequent steps of the RCT pathway. Hence, ABCA1 catalyzes the rate-limiting step in the RCT pathway in HDL particle formation. ABCA1 either directly or indirectly mediates the active transport of cholesterol and PL across cellular membranes, where they are removed from cells by apoproteins. It appears that ABCA1 preferentially mediates the removal of cholesterol from late endosomes/lysosomes (73, 74) that would otherwise be esterified by ACAT (58, 75). Cellular cholesterol accumulation leads to increased ABCA1 expression (64, 76) through oxysterol-dependent activation of liver X receptor (LXR), a nuclear receptor that binds to the promoter region of the ABCA1 gene to activate its expression (77-79). The activity of the ACAT enzyme is dependent on the pool of cellular FC, and it likely functions alongside ABCA1 to maintain cholesterol balance and to limit the concentration of toxic FC within cells. Consequently, increased ABCA1 expression correlates with increased cholesterol efflux to HDL apoproteins (80, 81).

The role of ABCA1 in HDL formation was further analyzed upon the creation of ABCA1 knockout (ABCA1-KO) mice. Three independent laboratories have reported that mice lacking ABCA1 had a near absence of plasma HDL (82-84). Interestingly, liver ABCA1 seems to play an important role in the generation of HDL particles, probably because it is a high expresser of ABCA1 (85) and is the major organ to synthesize apoA-I (86, 87). Mice with liver-specific deficiency of ABCA1 had 80% lower plasma HDL-C levels; thus, it is thought that hepatic ABCA1 is critical for the initial lipidation of newly secreted lipid-poor apoA-I to form nascent discoidal (pre-β-migrating) HDL, protecting it from rapid degradation and allowing it to go on to form mature HDL (88). Some evidence also suggests hepatic ABCA1 shunts cholesterol out of the VLDL assembly pool and into the HDL formation pool (89). Surprisingly, mice with macrophage-specific deficiency of ABCA1 had little effect on plasma HDL-C levels, but were susceptible to atherosclerosis (90-92). These results suggest cholesterol efflux from macrophage foam cells plays an important role in the protection against atherosclerosis, but not in determining plasma HDL-C levels.

1.5.3 Interactions between apoA-I and ABCA1

Increased ABCA1 expression correlates with increased cell surface binding of apoA-I (93, 94). Currently, there are two models to explain the interaction between apoA-I and ABCA1 to mediate lipid efflux [reviewed in (95)]. The first model suggests apoA-I does not interact with ABCA1 directly, but rather with plasma membrane (PM) lipid domains created by ABCA1 (9). The second proposes a direct binding interaction in which apoA-I and ABCA1 bind in a ligand-receptor manner to promote cholesterol and PL efflux (95).

The first model (**Figure 1.6A**) proposes that functional ABCA1 generates an unstable lipid domain by flipping PL, primarily phosphatidylserine (PS), to the outer leaflet of the PM (96).

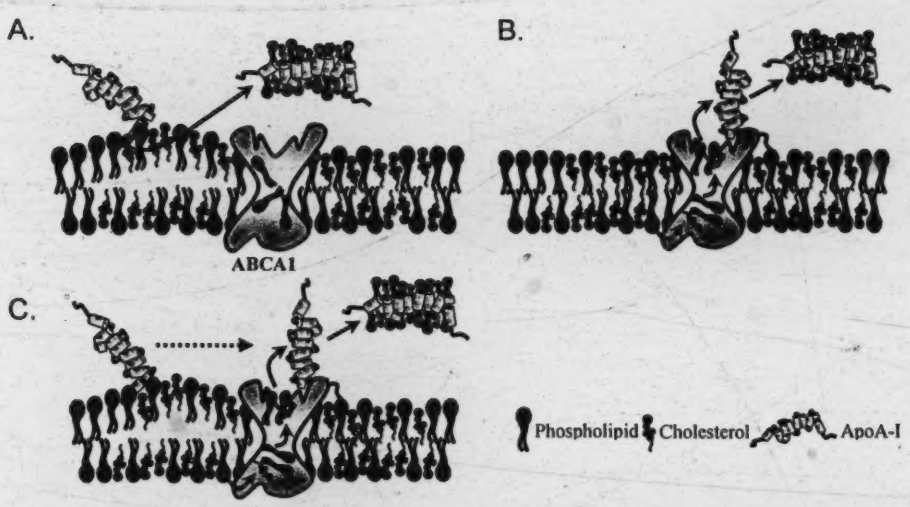

Figure 1.6: Proposed models for ABCA1-mediated lipid efflux to apoA-I. (A) ApoA-I associates with a specialized membrane domain formed by the action of ABCA1 and extracts membrane lipids. (B) ApoA-I binds directly to ABCA1 and acquires both cholesterol and phospholipids to form HDL. (C) Helix 10 of apoA-I tethers apoA-I to a specialized membrane domain formed by ABCA1. ApoA-I diffuses laterally along the membrane bilayer and complexes with ABCA1 to accept lipids. This figure was published in (95).

It has been observed that ABCA1-expressing cells have membrane structure perturbations that could be enriched in PL and cholesterol in the outer leaflet of the PM (97, 98). This unstable microenvironment on the cell surface is thought to allow the amphipathic apoA-I to readily interact with and solubilize the membrane lipids, leading to cellular lipid removal upon dissociation of the lipidated protein from the membrane (99, 100). A study using a fluorescence photobleaching technique demonstrated that membrane-associated apoA-I has diffusional properties that are consistent with an interaction with membrane lipids rather than with a membrane protein (101). Interestingly, a recent study suggested that ABCA1 mediates the formation of different lipid domains that contain their own unique lipid composition on the PM such that ABCA1 mediates the formation of different HDL species depending on which domain apoA-I interacts with on the PM (102).

Studies with ABCA1 mutants have also helped us to understand the nature of the molecular interaction between apoA-I and ABCA1. Chambenoit *et al* showed that apoA-

I cell surface binding is dependent on a fully functional ABCA1 protein, because cells expressing an ATPase-defective ABCA1 fail to bind apoA-I (101). It was concluded that cells lacking functional ABCA1 would not be able to flip PS to the outer leaflet of the plasma membrane, thereby inhibiting the generation of a suitable lipid environment required for apoA-I docking. In support of this is the observation that the expression of ABCA1 correlates with the amount of exofacial PS (101) and the requirement for PS flipping to release cellular phospholipids to apoA-I (103). However, another study demonstrated that apoptosis-induced exofacial PS flipping was not sufficient to stimulate lipid efflux to apoA-I (104). A more recent study showed treatment of ABCA1-expressing human fibroblasts with phosphatidylcholine-specific phospholipase C or sphingomyelinase had no effect on the ability of apoA-I to associate with ABCA1, suggesting that alterations in plasma membrane PL content by ABCA1 do not predict apoA-I binding to cells (105).

Studies using chemical cross-linkers have suggested that apoA-I binds directly to cell surface ABCA1 to form a high affinity complex, and that this binding is required for ABCA1-mediated lipid efflux (93, 98, 106) (**Figure 1.6B**). ApoA-I has been shown to cross-link to ABCA1 in an aqueous environment (107) and at a distance as little as 3 Å away, favoring a very close association between the two proteins (108). One ABCA1 transporter seems to bind to one molecule of apoA-I (109). In support of the direct association model, inhibition of ABCA1 by glybenclamide has been shown to inhibit both lipid efflux and cross-linking of apoA-I to ABCA1, suggesting a close relationship between apoA-I cross-linking and lipid efflux (94). Studies with ABCA1 mutants have also provided mounting evidence for the direct binding of apoA-I to ABCA1. Fitzgerald *et al.* have shown that four naturally occurring ABCA1 mutants harboring missense mutations in the two extracellular loops, but which remain properly targeted to the PM, fail to promote cholesterol efflux and have diminished ability to cross-link to apoA-I (107). This implies that a direct interaction between apoA-I and ABCA1 extracellular loops is necessary for efflux. Interestingly, another ABCA1 mutant (W590S) that fails to promote lipid efflux but retains full cross-linking ability with apoA-I has been identified, suggesting that apoA-I binding is necessary but not sufficient to stimulate cholesterol efflux (107). Further studies with the W590S mutant revealed that apoA-I binds and

dissociates from the defective ABCA1 transporter at a rate similar to wildtype ABCA1, but the dissociated apoA-I was without lipids, implying that dissociation is not dependent on lipid transfer to apoA-I (106). An explanation for the inability of the W590S mutant to efflux lipids may be its inability to drive the flipping of PS to the exofacial side of the PM, proposed to be a requirement for lipid efflux in the indirect association model (103). Hence, the apoA-I/ABCA1 interaction is complex and may involve the association of apoA-I with lipid domains created by ABCA1, and/or ABCA1 directly.

Panagotopulos and colleagues observed that helix 10 of apoA-I is crucial for apoprotein-mediated cholesterol efflux, and that two apoA-I mutants containing intact helix 10 exhibited markedly reduced lipid-binding affinity, but promoted cholesterol efflux much like wildtype apoA-I (110). To explain this observation, the authors propose a hybrid model integrating the protein-protein and lipid domain interaction hypotheses. They speculate that helix 10 functions to tether apoA-I to the ABCA1-generated lipid domain such that apoA-I can diffuse laterally until it comes into contact with ABCA1 to form a productive complex, leading to the lipidation of apoA-I (110). This hybrid model (**Figure 1.6C**) would explain why apoA-I does not readily cross-link to ABCA1 at lower temperatures, as the lack of membrane fluidity would prevent the initial insertion and lateral diffusion of apoA-I (107).

1.5.4 Protection by apoA-I against calpain-dependent proteolysis of ABCA1

Another remarkable facet of the apoA-I-ABCA1 interaction is the demonstration that HDL apoproteins serve to protect ABCA1 against proteolysis, thereby increasing ABCA1 activity and further facilitating apoprotein-mediated lipid efflux (111-113). Arakawa and Yokoyama demonstrated that incubation of apoA-I with cultured THP-1 cells increased ABCA1 protein levels without increasing ABCA1 message; free apoA-II did the same thing, however intact HDL particles did not (111). Thiol-protease inhibitors could also prevent ABCA1 degradation while enhancing cholesterol efflux, suggesting a possible mechanism for apoproteins to stabilize ABCA1 (111). Wang and colleagues subsequently reported that calpain-dependent proteolysis is involved in the degradation of ABCA1 via a proline, glutamate, serine, and threonine (PEST)-rich intracellular sequence of the transporter, and that deletion of this PEST sequence or treatment of cells with

apoA-I could inhibit this proteolysis and increase cell surface ABCA1 (112). They also demonstrated that infusion of apoA-I increased ABCA1 protein levels in the liver and peritoneal macrophages of C57BL/6J mice (112). This group subsequently reported that phosphorylation of threonine residues within this PEST sequence is required for calpain-dependent proteolysis of ABCA1, and apoA-I reverses this phosphorylation. In this way, the authors propose that apoA-I modulates ABCA1 expression by directly binding to ABCA1, or a membrane alteration secondary to ABCA1-mediated PL efflux could lead to decreased phosphorylation of the PEST sequence to increase ABCA1 activity (112, 114). Alternatively, the authors could not exclude the possibility that apoA-I binding or PL efflux induces a signaling cascade that leads to altered kinase or phosphatase activity and hence phosphorylation of the PEST sequence (113). Further studies suggest the PEST sequence of ABCA1 is required for the internalization of the transporter, to mediate efflux of the portion of total cholesterol efflux derived from intracellular pools (115).

1.5.5 Additional mechanisms of cholesterol efflux to HDL

Following the initial lipidation of apoA-I by ABCA1, discoidal (pre-β-migrating) nascent HDL particles are thought to receive additional cell cholesterol through the actions of other membrane transporters including ATP-binding cassette transporter G1 (ABCG1) and SR-BI, and by a passive diffusion mechanism. Mature HDL particles can also participate in ABCA1-mediated cholesterol efflux, but this is dependent on their ability to release lipid-free exchangeable apolipoproteins (such as apoA-I or apoE) from the particle surface (116). ABCG1 is a cell membrane transporter that facilitates the transfer of cholesterol from cells, including macrophages, to larger, spherical (α-migrating) HDL particles (117, 118). ABCG1-null mice fed a high-fat and high-cholesterol diet accumulated large amounts of cholesterol in macrophages and the liver without any change in plasma lipids or lipoprotein levels, indicating a role for ABCG1 in cholesterol homeostasis, but not necessarily in predicting plasma HDL-C levels (119). SR-BI facilitates the bidirectional transfer of FC between cells and mature HDL. This process is dependent on a concentration gradient, whereby a net transfer of cholesterol onto HDL particles is driven by the formation of a concentration gradient between the

HDL particle and the cell PM when LCAT esterifies FC on the surface of the HDL particle (39). Although still unclear, it is assumed that SR-BI accelerates the diffusion mechanism of FC efflux. Mature spherical HDL particles can also accept free cholesterol from cells via passive aqueous diffusion without assistance from membrane proteins (39). In this process, FC from cell membranes is spontaneously released into the aqueous phase where it incorporates onto spherical HDL particles. This mechanism is bidirectional, and, like SR-B1-mediated cholesterol efflux, is dependent on a concentration gradient of higher FC content on the cell surface down to the lower FC content on the HDL surface.

1.6 Regulation of cholesterol efflux by the Liver X Receptor

1.6.1 Regulation of ABCA1, cholesterol efflux, and reverse cholesterol transport by LXR

Transcription of ABCA1 is markedly enhanced by overloading cells with cholesterol. LXRα and LXRβ are nuclear receptors that maintain cholesterol homeostasis by sensing excess cellular cholesterol. Induction of ABCA1 transcription occurs by way of LXRα and/or LXRβ and the retinoid X receptor (RXR) (77, 78). LXR and RXR form obligate heterodimers that bind to response elements within the ABCA1 gene promoter region, thereby controlling its transcription. Oxysterols and retinoic acid bind to, and activate LXR and RXR, respectively (120); thus, treatment of cells with either an oxysterol or retinoic acid induces ABCA1 expression (77, 78). However, cholesterol delivered to cells must first be converted to oxysterols before inducing ABCA1 transcription. Hence, the series of events that leads to ABCA1 induction by LXR include the uptake of excess cholesterol by cells, the oxidation and generation of oxysterols by cytochome P450 enzymes, and finally, oxysterol activation of LXR to drive the transcription of ABCA1. In addition to conversion of FC to CE, cells are thus protected against accumulation of excess FC by increased expression of ABCA1, which mediates the transport of excess cholesterol to extracellular acceptor apoproteins. Thus, one can envision a possible role for LXR in mediating cholesterol efflux from peripheral sites such as macrophage foam cells, thereby enhancing RCT and protecting against atherosclerosis. Indeed, *in vitro* studies using a potent selective LXRα/β agonist (acetyl-podocarpic dimer) resulted in increased ABCA1 mRNA expression with increased cholesterol efflux to apoA-I upon treatment of THP-1 and human primary macrophages

with the agonist (121). *In vivo* studies with synthetic LXR agonists have also shown promising results with respect to stimulating RCT. Joseph *et al* showed that treatment of LDLr-knockout (LDLr-KO) and apoE-KO mice with the synthetic LXR agonist GW3965 resulted in the upregulation of ABCA1 protein in the macrophages of aortic lesions, and consequently reducing atherosclerotic development by 35-50% (122). Recently, verification that LXR activation can stimulate RCT was documented when LDLr/apobec-1 double KO mice were injected intraperitoneally with ^{3}H-cholesterol-labeled and cholesterol-loaded macrophages and monitored for the appearance of ^{3}H-tracer in plasma and feces. Administration of GW3965 significantly increased the levels of ^{3}H-tracer in plasma and feces in these mice, indicating stimulation of RCT from macrophages (123). Upregulation of ABCA1 expression mediated by LXR activators is associated with anti-atherogenic effects (124) but is complicated by fatty liver and hypertriglyceridemia (125, 126), due to the fact that LXR also positively controls the transcription of hepatic genes involved in fatty acid and TAG synthesis, including stearoyl CoA-desaturase 1, fatty acid synthase and sterol response element binding protein 1c (127). Therefore, the potentially dangerous side effects of a fatty liver as well as increased plasma TAG levels need to be addressed before LXR agonists can be considered viable therapeutic agents.

1.7 Oxidized lipoproteins in atherosclerosis

1.7.1 Introduction to lipoprotein oxidation

A large body of evidence has suggested that oxidation of lipoproteins, in particular LDL, contributes to the initiation and/or progression of atherosclerosis [reviewed in (128)]. Numerous studies have shown that oxidation of LDL particles induces macrophage foam cell formation, and subsequent atherosclerotic lesion evolution (129-131). Oxidized LDL is thought to induce atherosclerosis by stimulating the release of cytokines, chemokines, and growth factors from the injured endothelial cell, thereby stimulating monocyte infiltration and smooth muscle cell migration and proliferation in addition to macrophage and endothelial cell apoptosis (5, 132). Uptake of oxidized or acetylated LDL by macrophages is mediated by scavenger receptors, which do not behave like the LDLr in that they are not downregulated upon increased LDL uptake. Scavenger receptors are multi-ligand receptors that can take up oxidized LDL in a rapid

and unregulated fashion (131, 133, 134), leading to massive cholesterol deposition in the form of CE droplets and foam cell formation.

LDL can be oxidized by metal ions, lipoxygenases, or myeloperoxidase (MPO) (**Figure 1.7**).

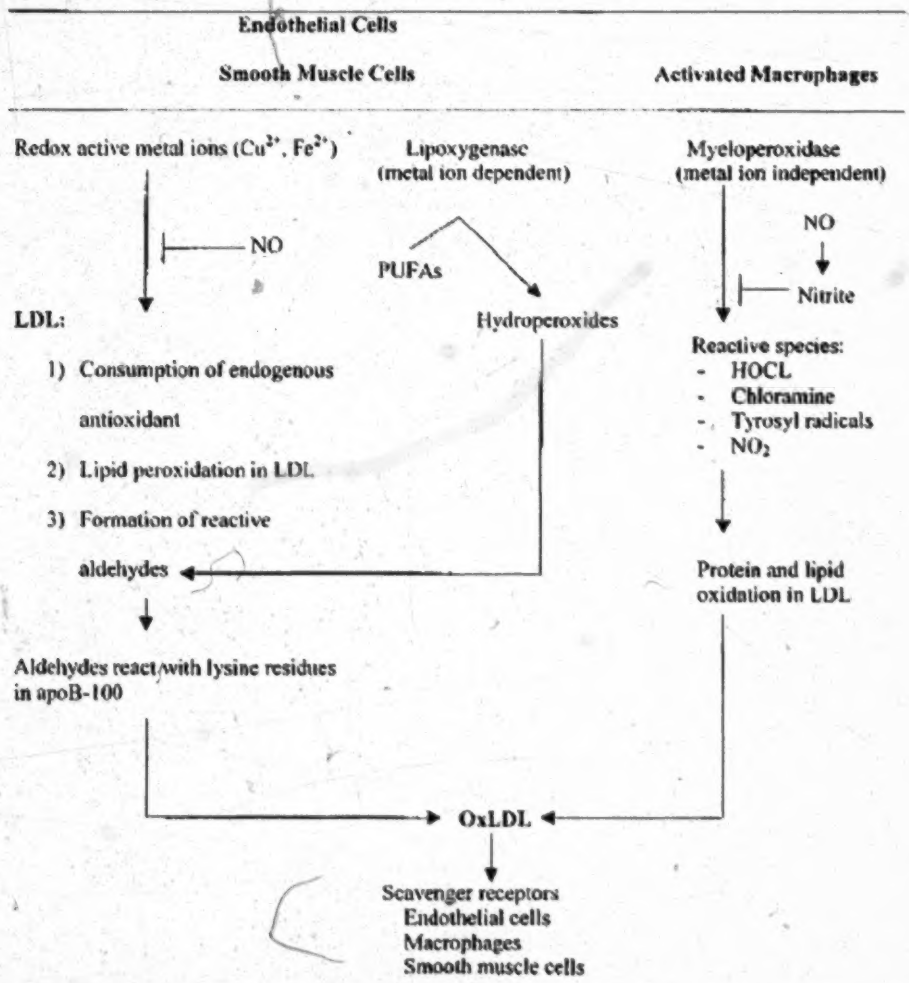

Figure 1.7: Mechanisms of LDL oxidation. Metal ions, lipoxygenase, and myeloperoxidase can oxidize LDL lipids and proteins. Oxidized LDL can then be taken up by peripheral cells via scavenger receptors. The figure was obtained from Mertens, A. and Holvoet, P. (132) with permission.

Free metal ions such as Cu^{2+} or Fe^{2+} can rapidly oxidize unsaturated fatty acids to lipid hydroperoxides, which are in turn converted to reactive aldehydes. Reaction of these aldehydes with positively charged ε-amino groups of lysine residues on apoB render LDL

more negatively charged. This results in decreased affinity for the LDLr and increased affinity for scavenger receptors (132). However, it is unlikely that free metal ions are responsible for *in vivo* oxidation, since plasma and interstitial fluid concentrations of these ions appear to be very low (135). Polyunsaturated fatty acids of LDL PL can be oxidized into lipid hydroperoxides *in vivo* by 15-Lipoxygenase, an enzyme produced by endothelial cells and monocytes/macrophages (**Figure 1.7**) (132). Another potential physiological catalyst of lipoprotein oxidation is MPO, a heme protein released from activated phagocytes (35). MPO uses H_2O_2 generated by activated monocytes, macrophages, and neutrophils as a substrate for oxidative reactions. Reactive species such as hypochlorous acid, chloramines, tyrosyl radicals, and nitrogen dioxide can be generated from MPO. These reactive species can oxidize lipids and protein of LDL, among other targets (**Figure 1.7**) (132).

Protein oxidation often leads to covalent intra- and inter-protein cross-linking (35, 136). Oxidation of proteins by systems utilizing peroxidases have been shown to create protein-protein cross-links between tyrosine residues, generating dityrosine in the oxidized target (136-138). Dityrosine is an intensely fluorescent molecule, and an increase in dityrosine fluorescence in these systems suggests that these protein-protein cross-links involve coupling of separate protein tyrosine residues. Upon binding of H_2O_2 to the heme group of MPO, the formation of dityrosine first involves the oxidation of tyrosine residues either free in solution or part of the polypeptide chain to create tyrosyl radicals. Two tyrosyl radicals can then react with each other to form dityrosine with its characteristic fluorescence. The products of tyrosyl radical oxidation are illustrated in **Figure 1.8**.

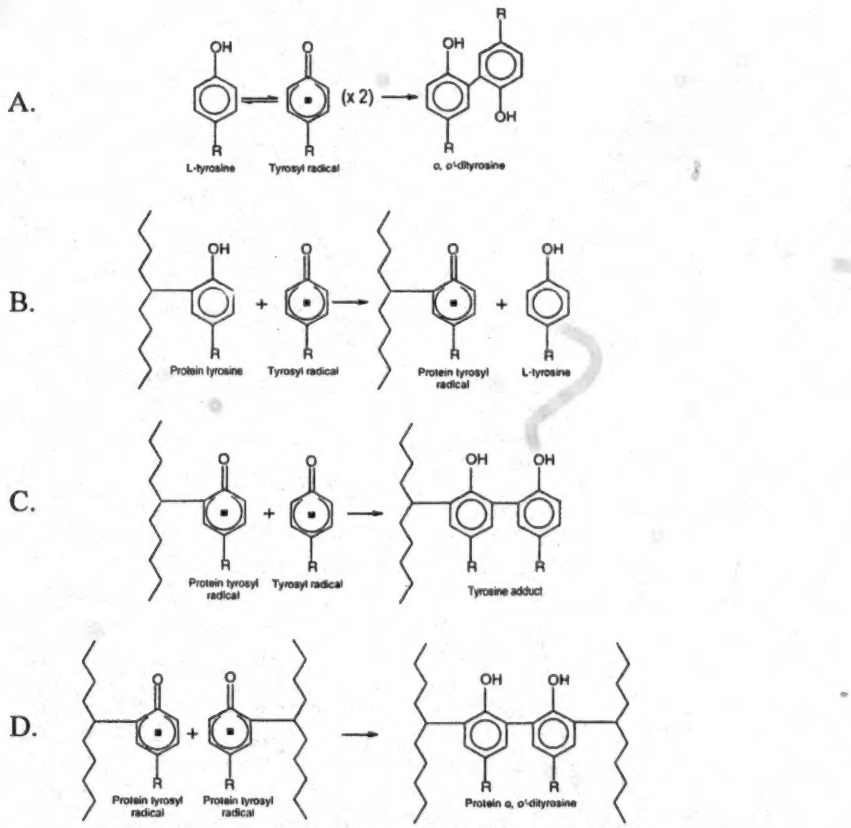

Figure 1.8: Products of peroxidase-mediated tyrosine oxidation. (A) Horseradish peroxidase, MPO, or other peroxidases convert free L-tyrosine to tyrosyl radical. 2 free tyrosyl radicals can undergo phenolic coupling to generate free dityrosine. (B) Alternatively, a free tyrosyl radical can interact with a protein tyrosine to generate a protein tyrosyl radical. The protein tyrosyl radical can either interact with a free tyrosyl radical to generate a tyrosine adduct (C), or 2 protein tyrosyl radicals can couple to form an inter-protein dityrosine cross-link (D). While tyrosyl radicals are short-lived, dityrosine is a stable product.

MPO is a likely candidate for the *in vivo* oxidation of lipoproteins (139). Physiologically, active MPO, which is capable of producing hypochlorous acid from free chloride ions, and tyrosyl radical from free L-tyrosine, is found in human atherosclerotic lesions (140). **Figure 1.9** illustrates the mechanism of free radical generation by activated phagocytes. Evidence for MPO as a physiological mediator of oxidation of lipoproteins is supported by the demonstration that proteins modified by hypochlorite, a

product expected to be generated only by MPO (141), is found in both early and advanced atherosclerotic lesions (142, 143).

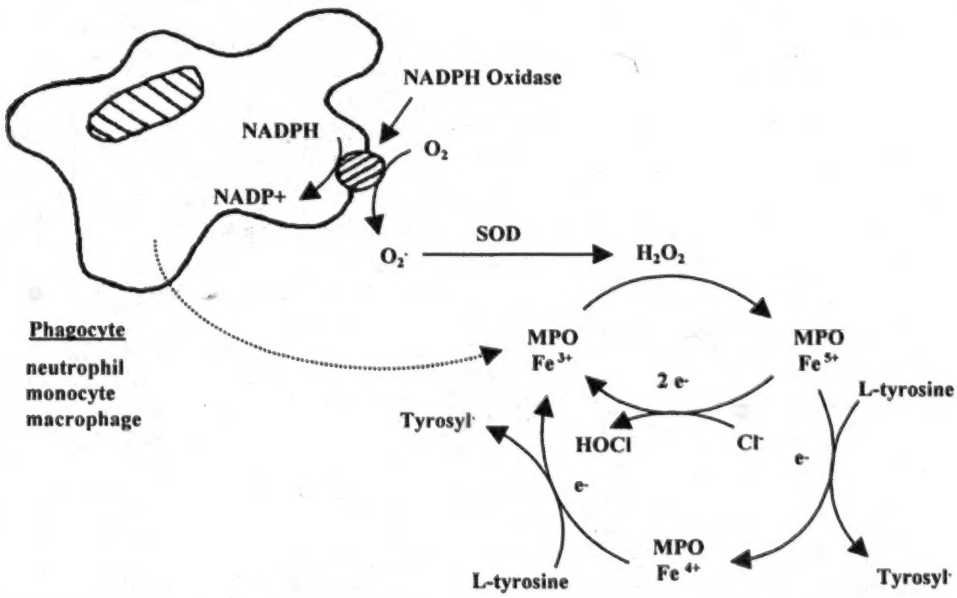

Figure 1.9: Model for tyrosyl radical generation in vivo. Activated human phagocytes (neutrophils, monocytes, and macrophages) secrete the enzyme myeloperoxidase (MPO) and generate superoxide (O_2^-) by the actions of the plasma membrane associated NADPH oxidase. Superoxide spontaneously dismutates or is enzymatically converted by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). H_2O_2 is used by MPO to catalyze the oxidation of its substrates to free radicals in the extracellular or interstitial fluid. These radicals carry out the actual oxidative damage characteristic of activated phagocytes. One substrate for oxidation by MPO is the amino acid L-tyrosine which is converted to tyrosyl radical (Tyroysl·). Cl^-, chloride ion; HOCl, hypochlorous acid; O_2, molecular oxygen; e^-; electron.

In addition, MPO-catalyzed oxidation products such as o,o'-dityrosine have been found in atherosclerotic lesion LDL in much higher levels than in circulating LDL, and there is a marked increase in dityrosine in early (fatty streaks) and advanced atherosclerotic lesions when compared to normal aortas (143, 144).

1.7.2 Role of oxidized HDL in cholesterol efflux

A number of studies have reported that HDL is readily oxidized *in vitro* and that HDL is actually more susceptible to oxidation than is LDL (35, 145). Reasons for this include the smaller size of HDL and therefore decreased capacity to carry antioxidant

molecules on the particle surface (35). Most studies have reported that oxidative modification of HDL impairs its ability to promote cholesterol efflux from cultured cells (146-154), thereby rendering it less effective as a cardioprotective particle. However, studies in our laboratory indicate oxidation of HDL by peroxidase-generated tyrosyl radical markedly enhances the ability of HDL to promote the removal of cholesterol from cultured cells (155-157).

1.7.3 Role of tyrosyl radical-oxidized HDL in cholesterol efflux and atherosclerosis

Studies from our laboratory suggest tyrosyl radical preferentially oxidizes the protein rather than the lipid components of HDL, with tyrosyl radical generating low levels of lipid oxidation products (156). Furthermore, tyrosyl radical can oxidize HDL apoproteins in their lipid-free state, thereby inducing oligomerization of the HDL apoproteins (**Figure 1.10**) (157). Thus, it appears that complexes between apoA-I and apoA-II, the two main proteins of HDL, can be formed in the presence or absence of lipids during oxidation by tyrosyl radical (155, 157). In contrast, oxidation of HDL by hydroxyl radical, lipoxygenase, or metal ions such as copper generates high levels of lipid peroxidation products, with modification of apoproteins occurring secondarily only after lipid oxidation (35). As evidence of this, in contrast to peroxidase-generated tyrosyl radical, copper ion failed to oxidize or induce oligomers of free HDL apoproteins in the absence of lipids (**Figure 1.10**) (35).

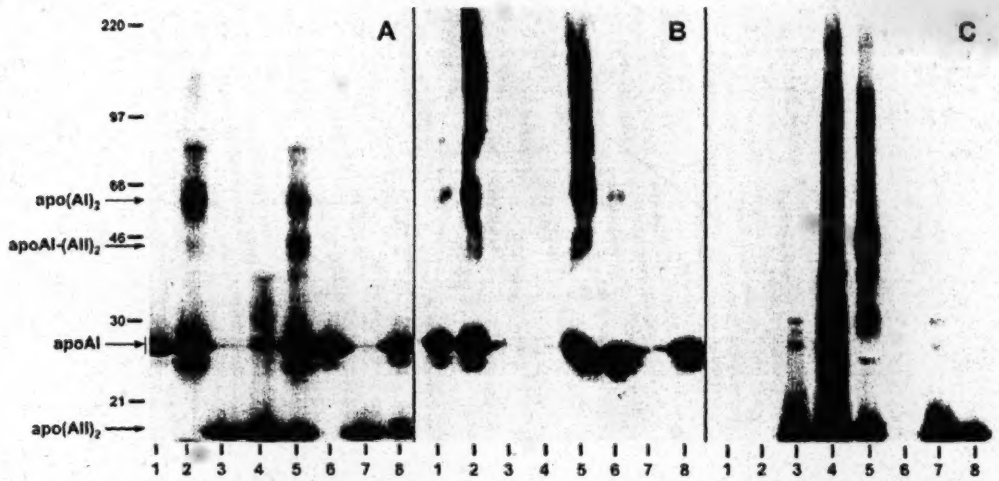

Figure 1.10: Oligomerization of free apoA-I and apoA-II by tyrosyl radical but not by copper ion. Apos were oxidized by either 2 μM copper ion, or tyrosyl radical as described in Chapter 2 *section 2.2.2*. (A) Non-reducing SDS-PAGE stained with Coomassie Blue; (B) anti-apoA-I Western blot; (C) anti-apoA-II Western blot. Lane assignments: 1, unoxidized apoA-I; 2, tyrosyl radical-oxidized apoA-I; 3, unoxidized apoA-II; 4, tyrosyl radical-oxidized apoA-II; 5, tyrosyl radical-oxidized apoA-I + apoA-II; 6, copper-oxidized apoA-I; 7, copper-oxidized apoA-II; 8, copper-oxidized apoA-I + apoA-II. While tyrosyl radical was able to promote oligomerization of the apoproteins in the absence of lipids, copper ion was not. The figure was obtained from Francis, G.A. and reprinted from (35) with permission from Elsevier.

1.7.3.1 Tyrosyl radical oxidation of HDL enhances cholesterol removal from cultured cells

HDL oxidized by the complete system of horseradish peroxidase (HRP)-H_2O_2-L-tyrosine (tyrosylated HDL or tyrHDL) has an enhanced ability to mobilize cholesterol from cells. Cultured human skin fibroblasts (HSF) treated with tyrHDL had an increased ability to promote the removal of cellular cholesterol available for esterification by ACAT (**Figure 1.11A and 1.12A**), and had significantly less CE and FC mass in the cells (**Figure 1.12B**), indicating that tyrHDL promotes increased cholesterol efflux (155).

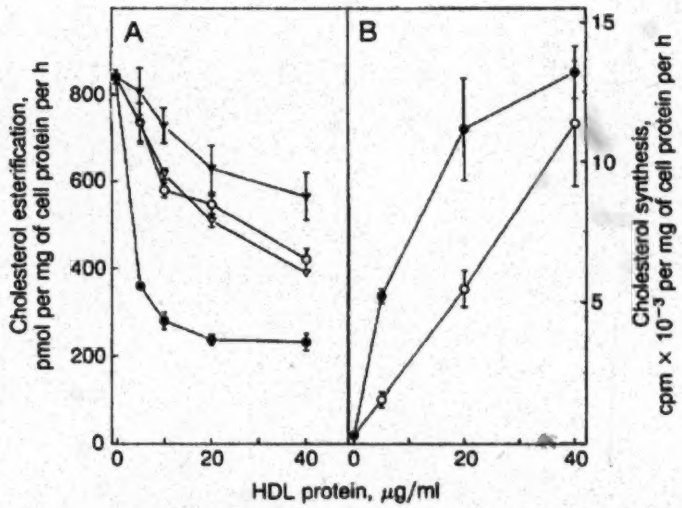

Figure 1.11: Cholesterol esterification and synthesis in fibroblasts incubated with tyrHDL. (A) Cholesterol loaded HSF were incubated for 16 h with the indicated concentrations of HDL. Then the cells were washed and incubated for 1 h with [^{14}C]oleate for incorporation into cholesteryl esters. Cellular lipids were extracted and assayed for cholesteryl [^{14}C]oleate. (B) Following 24 h exposure to HDL samples, cells were exposed to [^{14}C]acetate for 2 h to measure new cholesterol synthesis. *White circles*, control HDL; *dark circles*, tyrHDL complete system; *white triangles*, HDL oxidized with complete system but with heat-inactivated HRP; *dark triangles*, HDL oxidized with complete system minus L-tyrosine. The figure was obtained from Francis, G.A. (155) with permission. Copyright (1993) National Academy of Sciences, U.S.A.

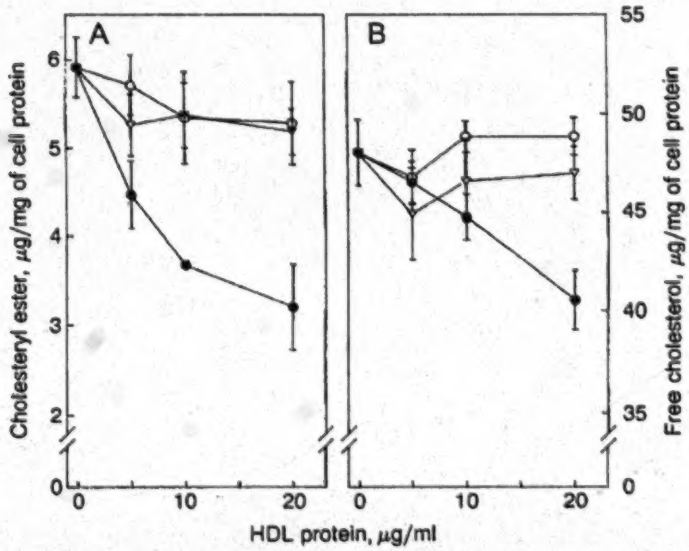

Figure 1.12: Cholesterol mass in fibroblasts coincubated with LDL and tyrHDL. HSF at 80% confluence were incubated for 48 h with 10% lipoprotein-deficient serum to upregulate LDLr activity. They were then incubated for 24 h in medium containing 50 μg/mL of LDL and the indicated concentration of HDL. (A) CE mass; (B) FC mass. *White circles*, control HDL; *dark circles*, tyrHDL complete system; *white triangles*, HDL oxidized with complete system but with heat-inactivated HRP. The figure was obtained from Francis, G.A. (155) with permission. Copyright (1993) National Academy of Sciences, U.S.A.

Similar results were seen in depletion by tyrHDL of CE and FC mass in cholesterol-loaded mouse peritoneal macrophages (155). This suggested that tyrHDL is able to deplete the regulatory pool of cholesterol that would normally be esterified by ACAT. Interestingly, efflux from this same pool of cholesterol is thought to be mediated by ABCA1 (58, 74, 75). Depletion of this regulatory pool of cholesterol was confirmed by the finding that HSF showed a marked increase in new cholesterol synthesis following treatment with tyrHDL (**Figure 1.11B**). The induction of ABCA1 that occurs with the overloading of cells with cholesterol is attributed to activation of the LXR/RXR heterodimer by oxysterols (77, 78). Our laboratory has demonstrated that tyrHDL is able to markedly deplete the regulatory pool of cholesterol even in cholesterol-depleted cells grown in lipoprotein deficient serum (Francis, G.A, unpublished data) – a condition that would yield minimal concentrations of cellular oxysterols. This indicates that tyrHDL may function to mobilize cholesterol via ABCA1 without requirement for LXR. The

effects of tyrHDL represented the first evidence of enhanced cholesterol mobilization by an oxidized form of HDL (155).

1.7.3.II TyrHDL increases the availability of cholesterol for new HDL particle formation

It was further demonstrated that tyrHDL was capable of mobilizing cholesterol for new HDL particle formation. When compared to normal HDL, cholesterol-loaded fibroblasts treated with low concentrations of tyrHDL showed depletion of CE and increased cell FC even prior to increasing cholesterol efflux to the culture medium, due to the absence of adequate concentrations of cholesterol acceptors in the medium (**Figure 1.13**).

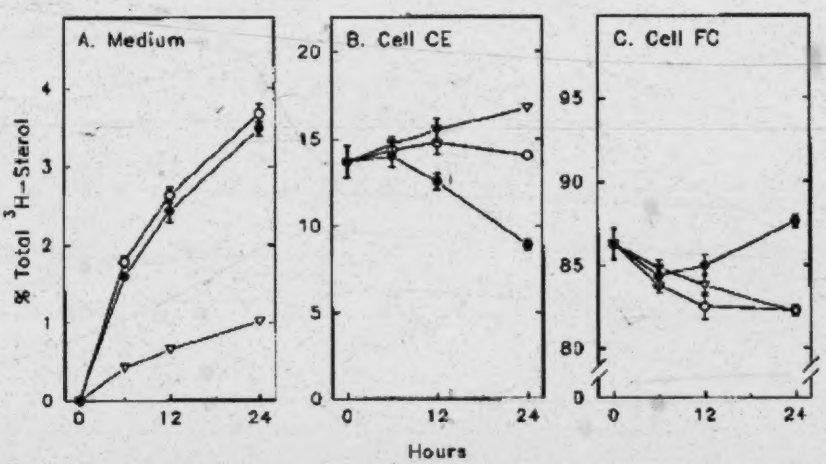

Figure 1.13: Time course of cholesteryl ester turnover and cholesterol efflux from cholesterol-loaded fibroblasts exposed to tyrHDL. Cells were labeled with [^{3}H]cholesterol during the last 40% of growth to confluence and then loaded with non-lipoprotein cholesterol for 48 h and equilibrated for 24 h in medium containing 1 mg/mL BSA (serum-free medium, SFM) to allow equilibration of cholesterol pools. The cells were then incubated with SFM (*white triangles*) alone, or the same medium plus 10 μg/mL HDL (*white circles*), or tyrHDL (*dark circles*). Results are expressed as percent of total (cell plus medium) [^{3}H]sterol in the medium (A), in cellular cholesteryl ester (CE) (B), and in cellular free cholesterol (FC) (C). This figure was obtained from Francis, G.A. and reprinted with permission from (156). Copyright (1996) American Chemical Society.

This occurred with no direct effect on the ACAT or neutral cholesteryl ester hydrolase enzyme activity (155, 156). TyrHDL had no effect on esterification by ACAT of exogenous cholesterol added to whole cell homogenates (155), nor did it inhibit the rate of hydrolysis of CE compared to HDL when the re-esterification step of the cholesteryl ester cycle was blocked by an ACAT inhibitor (156). These results indicate tyrHDL impacts the cholesteryl ester cycle by removing cholesterol available for esterification within the cycle. The results also indicated tyrHDL moves cholesterol from sites available for esterification by ACAT to sites available for removal from the cell by exogenous acceptors (the "efflux-accessible" pool), and that this can occur prior to depletion of cholesterol from the PM.

Subsequent studies found that there was no difference in the ability of tyrHDL or control HDL to stimulate passive FC efflux from the PM of cells, yet HSF pre-treated with tyrHDL showed increased cholesterol efflux to apoA-I (156). This is further evidence that tyrHDL has an enhanced ability to mobilize intracellular cholesterol to PM domains for subsequent removal by acceptor particles and suggests that tyrHDL can stimulate new HDL particle formation. This mechanism for diverting cholesterol from the ACAT substrate pool to an efflux-accessible pool without any change in passive cholesterol efflux suggests that it is the active efflux pathway that is upregulated in this system. This has far reaching implications in that ABCA1 is thought to mediate the active redistribution and efflux of cellular cholesterol to acceptor molecules. Therefore, tyrHDL may have an enhanced capacity to stimulate ABCA1-mediated FC efflux to increase plasma HDL levels, thereby protecting against atherosclerosis.

1.7.3.III Administration of tyrHDL increases plasma HDL-C and inhibits the development of atherosclerosis in apoE-deficient mice

Indeed, *in vivo* studies have shown that administration of tyrHDL in apoE-KO mice increases HDL-C and inhibits the development of atherosclerosis (158). Isolated native apoE-free HDL from C57BL/6 mice that were oxidized by tyrosyl radical showed apoprotein oligomerization and had an enhanced ability to deplete cultured fibroblasts of their regulatory pool of cholesterol available for esterification (158). When apoE-KO mice were injected intraperitoneally with either saline, 150 μg/mL of control HDL, or the

same amount of tyrHDL twice weekly for 8 weeks, the animals injected with tyrHDL had a maximum 2.3 fold increase in endogenous HDL-C, which fell toward the end of the treatment period. Despite similar rates of clearance of injected HDL particles as well as the drop in endogenous HDL-C after 8 weeks of treatment, the tyrHDL treatment resulted in 37% less aortic lesion development than in control HDL-treated mice and 67% less than in saline-injected animals (**Figure 1.14**) (158).

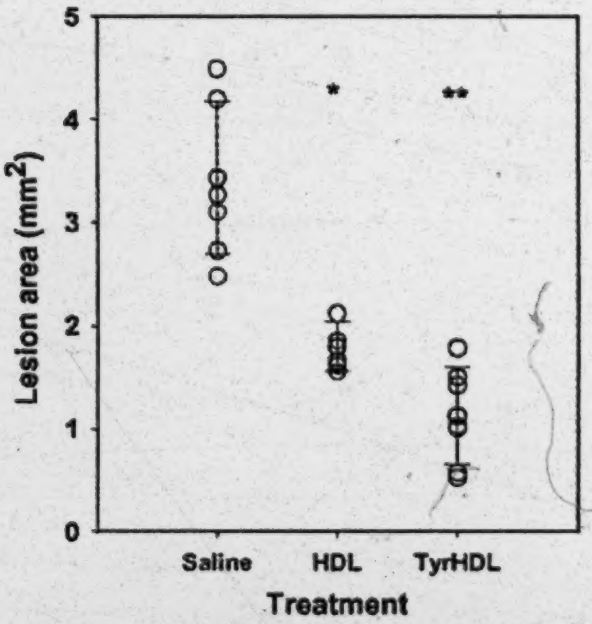

Figure 1.14: Atherosclerotic lesion areas in aortas from apoE-deficient mice injected with saline or treated with control HDL or tyrHDL for 8 weeks. Aortas were removed, cleaned, and opened longitudinally, and lesion area was determined from en face photographs. *Significantly different from saline-treated animals ($P<0.001$); **Significantly different from saline- and control HDL-treated animals ($P<0.001$). The figure was obtained from Macdonald, D.L. *et al* (158) with permission. Copyright (2003) Lippincott Williams & Wilkins.

1.7.3.IV Enhanced cholesterol mobilization by tyrHDL is mediated by apoA-I-A-II heterodimers

It was shown that the active components of tyrHDL are apoA-I-A-II heterodimers. Based on SDS-PAGE gels of tyrHDL preparations, our laboratory had observed the presence of oligomeric complexes of apoA-I and apoA-II in addition to apoAI-AII heterodimers (155).. To determine whether the protein or lipid components of tyrHDL were responsible for the enhanced mobilization of cholesterol from cells, the whole protein and lipid fractions of tyrHDL and HDL were isolated and then recombined into spherical or discoidal reconstituted HDL particles (rHDL) to test their activity (157). Spherical, and to a lesser extent, discoidal, rHDL containing the whole apoprotein fraction of tyrHDL had the same ability as intact tyrHDL to enhance cholesterol redistribution from fibroblasts when reconstituted with the whole lipid fraction of either HDL or tyrHDL (**Figure 1.15)** (157).

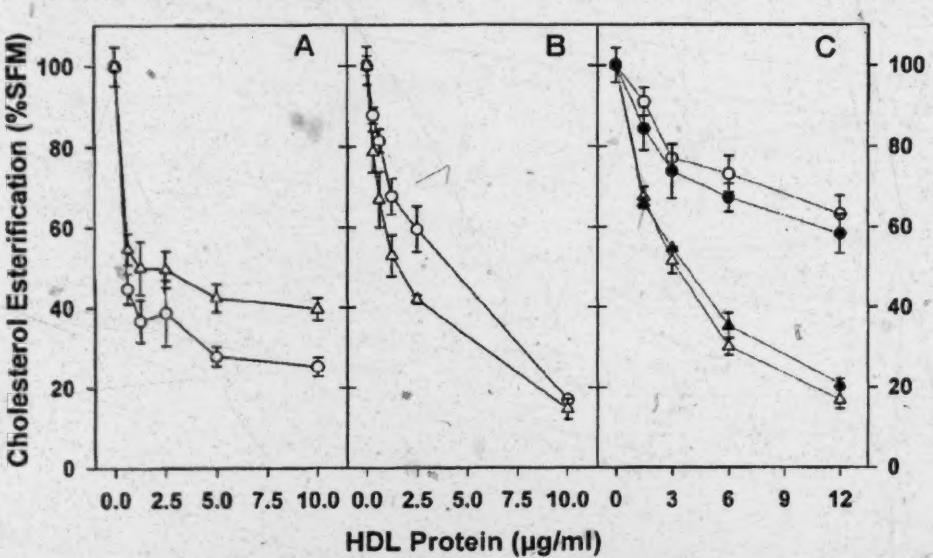

Figure 1.15: Reduction in cholesterol esterification by protein and lipid components of tyrHDL. (A) Lipid-free apoproteins of HDL (*circles*) or tyrHDL (*triangle*); (B) discoidal rHDL made using phosphatidylcholine and FC; (C) spherical rHDL made using the whole lipid fraction of HDL (*white symbols*) or tyrHDL (*dark symbols*). *Circles* are rHDL made from apoproteins of HDL, and *triangles* are from apoproteins of tyrHDL. Cholesterol esterification was measured after 16 h of incubation with apoproteins or rHDL as described in **Figure 1.11A**. Esterification was significantly less only when tyrosylated apoproteins were reconstituted with the whole lipid fraction of HDL. The figure was obtained from Wang, W.Q. *et al* (157) with permission.

The lipid-free protein fraction of tyrHDL failed to enhance the mobilization of cholesterol compared to lipid-free control HDL proteins, suggesting that optimal activity of tyrHDL proteins requires a specific conformation of tyrHDL apoproteins when present on the surface of spherical HDL particles. When the whole lipid fraction of tyrHDL particles was reconstituted with the whole protein fraction of HDL, this particle also failed to show enhanced capacity to deplete cells of cholesterol for esterification by ACAT (157). Furthermore, tyrosyl radical oxidation of free apoA-I or apoA-II in their lipid free form or in rHDL had no enhanced activity, whereas oxidation of apoA-I+apoA-II-containing HDL particles or a combination of lipid-free apoA-I and apoA-II oxidized and then reconstituted with lipids to make rHDL reproduced the activity of intact tyrHDL in depleting cholesterol available for esterification (157). Confirmation that apoA-I-A-II heterodimers are responsible for the activity of tyrHDL was obtained when apoA-I-A-II heterodimers were isolated from tyrHDL by way of preparative electrophoresis, and reconstitution of these heterodimers into spherical HDL particles resulted in marked increase in the ability of these particles to mobilize cholesterol for efflux (157).

1.8 Hypothesis and specific aims

In summary, research in our laboratory has previously shown that tyrHDL has a much greater capacity than native HDL to promote the removal of cholesterol from cells to apoA-I for new HDL particle formation. The active component of tyrHDL appears to be apoA-I-A-II heterodimers. *In vivo* studies using an animal model of atherosclerosis, the apoE-deficient mouse, showed that administration of tyrHDL yielded increased endogenous HDL-C levels and reduced development of atherosclerosis in those mice. ABCA1 mediates the mobilization and removal of cellular cholesterol that is thought to be acted upon by ACAT. The fact that tyrHDL is able to markedly enhance the depletion of the regulatory pool of cholesterol that would normally be esterified by ACAT, when compared to control HDL, strongly suggest that tyrHDL functions by way of ABCA1 expression and/or activity. Hence, we hypothesized that tyrHDL enhances the mobilization of cellular lipids for HDL formation by enhancing the expression or activity of ABCA1.

The objectives of this thesis are:

1. To determine whether tyrHDL increases ABCA1 mRNA and/or protein levels in human skin fibroblasts (HSF).

2. To determine whether tyrHDL can increase cell surface expression of ABCA1.

3. To determine whether tyrHDL enhances ABCA1 activity or expression in an LXR-dependent pathway.

4. To determine whether the enhanced ability of tyrHDL to promote cholesterol mobilization from cells involves a direct binding interaction with ABCA1.

5. To determine the effect of tyrHDL on production of HDL particles as assessed by 2-dimensional gel electrophoresis.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Cholesterol, L-tyrosine, horseradish peroxidase (HRP), hydrogen peroxide (30%, ACS grade), diethylenetriamine pentaacetic acid (DTPA; free acid form), liver X receptor agonist TO-901317, and essentially fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich. [^{14}C]Oleate (55 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Chelex 100 resin was from Bio-Rad. Dithiobis(succinimidyl propionate) (DSP) was purchased from Pierce. The organic solvents used in this study were purchased from Fisher Scientific. Dulbecco's modified Eagle's medium (DMEM) was purchased from BioWhittaker and fetal bovine serum (FBS) from Hyclone. Nitrocellulose membranes, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reagents, and pre-stained protein molecular mass markers were purchased from Bio-Rad.

2.2 Methods

2.2.1 Preparation of lipoproteins and apoA-I

HDL_3 (d = 1.125–1.21 g/ml, hereafter referred to as HDL) was isolated by standard ultracentrifugation techniques from the pooled plasma of healthy volunteers (159). HDL fractions were subjected to heparin-Sepharose affinity chromatography to remove apoE- and apoB-containing particles (160). The whole protein fraction of HDL was obtained by delipidating HDL and purified apoA-I obtained by FPLC as described (161). In detail, HDL (0.2 mL) was delipidated by suspension in 12 mL of hexane/isopropanol (3:2 v/v) for 30 min and then spun at 2000 rpm at 4°C for 10 min to pellet HDL apoproteins. Isolation of HDL apoproteins was performed by FPLC using Q-Sepharose Fast Flow (GE Healthcare) anion-exchange FPLC column (2.6 x 40 cm) with a two buffer system (buffer A: 20 mM Tris, 6 M Urea, pH 8.5, and buffer B: 20 mM Tris, 6 M Urea, 2M NaCl, pH 8.5) using the following gradient program:

Total flow volume (mL)	% buffer B
0.0	0.0
188.5	0.0
188.6	4.0
400.5	4.8
730.5	7.0

730.6	100
788.5	100
788.6	0

fractions were collected and subjected to SDS-PAGE and silver staining to visualize fractions containing apoA-I. These fractions were pooled and dialyzed in 50 mM ammonium bicarbonate, 1 mM EDTA at 4°C. Purified apoA-I was lyophilized and stored at -80 °C. ApoA-I was dissolved in 66 mM phosphate buffer containing 0.1 mM DTPA (pH 8.0) at a concentration of 1 mg/ml before use.

2.2.2 Modification of HDL by peroxidase

Tyrosyl-radical oxidation of 1 mg/mL of HDL was carried out at 37°C for 16-20 h in 66 mM potassium phosphate buffer (pH 8.0) containing 100 μM DTPA, 100 nM HRP (Boehringer Mannheim), 100 μM H_2O_2, and 100 μM L-tyrosine. These conditions had previously been determined to optimize dityrosine synthesis in HDL proteins (155). The phosphate buffer was passed over Chelex 100 resin to remove transition metal ions potentially able to catalyze lipoprotein oxidation, and DTPA was included to inhibit metal-ion-catalyzed oxidation reactions. Protein-associated dityrosine production was monitored by fluorescence spectrometry using excitation and emission wavelengths of 328 nm and 410 nm, respectively.

2.2.3 Trypsinization of HDL and tyrHDL

Trypsin modification of HDL and tyrHDL was performed as described (162). Briefly, HDL or tyrHDL (to a final concentration of 1 mg protein/mL in 66 mM phosphate buffer pH 8.0) were incubated with trypsin (Gibco BRL) at an HDL/trypsin protein ratio of 40:1, where 1/10 of the total volume of reaction mixture was 0.10 M Tris/0.01 M $CaCl_2$ at pH 8.0. The $CaCl_2$ activates the trypsin. The mixture was incubated at 37°C for 30 min. After incubation, samples were chilled on ice and trypsin activity was inhibited by addition of phenylmethylsulfonylfluoride (1 mM). HDL and tyrHDL was separated from trypsin and cleaved peptides by chromatography on a 1×30 cm Sephadex G-75 column. This procedure digests ~15% of the total HDL protein content of HDL without disturbing its lipid composition. The phospholipid content of the

trypsinized HDL particles was analyzed, and HDL concentrations used to treat cells were based on HDL phospholipid content rather than HDL protein concentrations.

2.2.4 Cell culture

Human skin fibroblasts (HSF) were obtained by punch biopsy from healthy human donors; some cell lines were a kind gift of Dr. John Oram, University of Washington. Human aortic smooth muscle cells (HASMC) were obtained from Dr. Russell Ross, University of Washington, and from the American Type Culture Collection (CRL-1999). Skin biopsies from LXRα and β double knockout C57BL/6 mice (a kind gift from Dr. David Mangelsdorf, University of Texas Southwestern) were collected in DMEM supplemented with 20% FBS. Tissue biopsies from wildtype and LXRα and β double knockout C57BL/6 mice were incubated in 21.75 mg/mL Dispase® (Invitrogen) dissolved in DMEM containing 50 units/ml penicillin, and 50 μg/ml streptomycin overnight at 4°C to facilitate removal of the epidermal layer. After removal of the epidermis, biopsies were cut into smaller pieces and washed 3 times with DMEM containing 20% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1X Fungizone® (Invitrogen). These smaller tissue pieces were then seeded into T-25 cm^2 flasks with four pieces of tissue per flask. Each of the tissue pieces was covered with DMEM containing 20% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1X Fungizone® and cultured in 95% air and 5% CO2 at 37°C. The conditions of isolation (particularly the 20% FBS) favor the growth of fibroblasts, which will usually out-compete any other cell types in growth. Once fibroblasts grew out from the tissues, the tissues were removed and fibroblasts were left to grow. Identification of fibroblasts was based on morphology (fibroblasts have a characteristic spindle shape). Mouse skin fibroblasts (MSF) were isolated and expanded as cell lines once they grew to confluence. All cell lines were cultured in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin in humidified 95% air and 5% CO_2 at 37°C unless specifically indicated.

To load cells with non-lipoprotein cholesterol, confluent cells were rinsed twice with phosphate-buffered saline (PBS) containing 1 mg/ml BSA (PBS/BSA) and incubated for 24 h in DMEM containing 2 mg/ml BSA with 30 μg/ml cholesterol added from a 10 mg/ml stock in ethanol. To allow equilibration of added cholesterol, cells were

rinsed twice with PBS/BSA and incubated for an additional 24 h in DMEM containing 1 mg/ml BSA (DMEM/BSA).

2.2.5 Cholesterol esterification assay

Cells were seeded on 16 mm wells and grown to confluence in DMEM containing 10% FBS. To assess the ability of HDL, tyrHDL, trypsinized HDL, or trypsinized tyrHDL to deplete cellular free cholesterol available for esterification by acyl CoA:cholesterol acyltransferase (ACAT), cells loaded with non-lipoprotein cholesterol and equilibrated were rinsed once with PBS/BSA, incubated for 16 h in DMEM/BSA, or same media plus 0-20 μg/ml HDL or tyrHDL protein, or 0-40 μM HDL, tyrHDL, trypsinized HDL or trypsinized tyrHDL phospholipid. Cells were then rinsed once with PBS, and incubated for 1 h more with DMEM containing 9 μM [^{14}C]oleate bound to 3 μM BSA (163). At the end of the incubation, cells were chilled on ice to prevent further incorporation of [^{14}C]oleate, rinsed twice with iced PBS/BSA and twice with iced PBS, and stored at –20 °C until lipid extraction. Cell layers were incubated at room temperature with 1 mL hexane:isopropanol (3:2, v/v) for 30 min to extract total cell lipids. These extracts were transferred to 13 x 100 mm glass tubes containing 20 μL of complete carrier as standards (0.5 mg/mL CE, 1 mg/mL FC, 1 mg/mL 1-monooleoyl-glycerol, 1 mg/mL 1,2 distearoyl-glycerol, 1 mg/mL triolein [TAG], and 1 mg/mL oleic acid). Cells were rinsed twice more in hexane:isopropanol, and these extracts added to the tubes. Total extracts were dried by gentle heating and air, and then resuspended in 110 μL of ice-cold chloroform. 100 μL of this was spotted on PE SIL G plastic-backed plates (Whatman), and the plates developed in hexane/diethyl either/acetic acid (130:40:1.5 v/v/v). Lipid spots were identified by staining with I_2 vapor. After allowing I_2 stain to evaporate, cholesterol ester spots were taken for determination of radioactivity by liquid scintillation counting.

Cell protein was extracted by incubation of cells with 0.5 mL of 0.1 N NaOH for 1 h on a rotary shaker. Total cell protein was determined by the Lowry assay (164) using BSA as standard.

2.2.6 Reverse transcriptase-PCR analysis of ABCA1 mRNA

Total RNA was isolated from cells by guanidine isothiocyanate/phenol/ chloroform extraction (165). The concentration of RNA was measured spectrophotometrically at a wavelength of 260 nm, and 2 μg of RNA was treated with DNase I (Invitrogen) following the manufacturer's guidelines. First strand cDNA synthesis was performed using 500 nM of oligo(dT) primer and Superscript™ RNase H (Invitrogen). Each reaction mixture contained 100 units of Superscript™ enzyme, 1x first strand buffer (50 mM Tris-HCl, pH 8.0), 0.5 μM dNTP mix, 0.01 M dithiothreitol, 0.05 μg/μl BSA, and 2 units of RNase inhibitor (Invitrogen). The mixtures were incubated at 45 °C for 90 min followed by incubation at 95 °C for 3 min (Whatman Biometra T-gradient thermocycler) and then put promptly on ice. Amplification of ABCA1 and cyclophilin mRNAs was performed in tandem to ensure equal amounts of starting cDNA for each sample. Diethyl pyrocarbonate-treated water, 1x PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 1.5 mM $MgCl_2$, 0.1 mM dNTPs, and cDNA were added to 200 μl of thin walled PCR tubes and mixed, and one-half volume was transferred to another PCR tube. One unit of Taq DNA polymerase (Invitrogen) and 1.5 μl of 10 μM forward and reverse primers (ABCA1 or cyclophilin) were added to complete the reaction mixture. ABCA1 amplification was performed by initially denaturing DNA at 95 °C for 3 min. Thereafter, denaturing was at 95 °C for 75 s, annealing at 54.6 °C for 75 s, and extension at 72 °C for 55 s for a total of 31 cycles with a final extension period of 5 min. Human cyclophilin amplification was performed using similar conditions except the annealing temperature was 48 °C with a total of 33 cycles. PCR products were electrophoresed on a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light. The primers used were as follows: human ABCA1, 5'-GAC ATC CTG AAG CCA ATC CTG A (forward), 5'-CCT TGT GGC TGG AGT GTC AGG T (reverse); human cyclophilin, 5'-ACC CAA AGG GAA CTG CAG CGA GAG C (forward), 5'-CCG CGT CTC CTT TGA GCT GTT TGC AG (reverse).

2.2.7 Western blot analysis of ABCA1 Protein

Cells were grown to confluence on 100 mm culture dishes in DMEM containing 10% FBS, then loaded with non-lipoprotein cholesterol or not, and then equilibrated.

Cells were then incubated with DMEM/BSA, or same media plus 10 μg/mL of apoA-I, HDL, or tyrHDL for 2 or 16 h. These time points were chosen to have an early and later time point of tyrHDL exposure. Crude membrane proteins were prepared by homogenizing cells on ice in 50 mM Tris-HCl buffer, pH 7.4, containing complete protease inhibitors (Roche Molecular Biochemicals) and 1 mM EDTA. The nuclear fraction was removed by centrifugation for 2 min at 5,000 rpm (2,040 x g), and the supernatant was subsequently centrifuged for 30 min at 14,000 rpm (16,000 x g). The pellet was then resuspended in the homogenizing buffer with 0.45 M urea, 0.1 % Triton X-100, 0.2 % SDS and 0.05 % dithiothreitol and after determination of protein content by a BCA method (Pierce) the membrane preparations were stored at -80 °C until use. Fifteen to thirty micrograms of membrane proteins were separated by 7.5-15% gradient SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane for 16 h at 4 °C. Immunoblotting was performed using a rabbit polyclonal antibody to ABCA1 (1:1000, Novus Biologicals) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000, Sigma-Aldrich). Briefly, membranes were blocked for 1 h at room temperature with 5% skim milk power in Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, pH 7.5) containing 0.1% Tween 20 (TBS/T), rinsed three times with TBS/T, and incubated for 1 h at room temperature or 16 h at 4 °C with primary antibodies diluted in TBS/T containing 1% skim milk powder. Following the incubation, membranes were rinsed five times with TBS/T and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were diluted in TBS/T containing 1% skim milk powder. Membranes were extensively rinsed with TBS/T before chemiluminescent detection using the enhanced chemiluminescence assay system (Amersham Biosciences). The blots were reprobed with anti-protein disulfide isomerase (PDI) antibody (StressGen Biotechnologies) to verify equal protein loading.

2.2.8 Measurement of total and cell surface ABCA1

HSF were grown to confluence on 100 mm culture dishes in DMEM containing 10% FBS, then loaded with non-lipoprotein cholesterol and equilibrated. Cells were then incubated with DMEM/BSA, or same media plus with 10 μg/mL of apoA-I, HDL, or

tyrHDL for 16 h. Cell surface proteins were biotinylated using a cell surface protein biotinylation and purification kit (Pierce) according to the manufacturer's protocol. The biotinylated samples were lysed in RIPA buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 4 mM EDTA) containing complete protease inhibitors (Roche Molecular Biochemicals). Cell protein concentration was determined using BSA as standard and 20-50 μg of total protein were run on 7.5-15% gradient SDS-PAGE to determine total ABCA1 levels by Western blot as described above. The blots were reprobed with anti-PDI antibody (StressGen Biotechnologies) to verify equal protein loading. To determine cell surface ABCA1 levels, biotinylated proteins were isolated from 0.5-0.8 mg of total cell proteins according to the manufacturer's protocol, and 50 μl of isolated samples were run on 7.5-15% gradient SDS-PAGE. Cell surface ABCA1 protein levels were determined by Western blot and the blots were reprobed with anti-heat shock protein 90 (HSP90) antibody (Sigma-Aldrich) to verify no contamination of cytosolic proteins in the purified samples. We found no HSP90 signal from the purified cell surface protein fraction, whereas HSP90 signal was detected from the blots of total cell protein.

2.2.9 Cross-linking of apoA-I, HDL or tyrHDL to ABCA1

Chemical cross-linking was performed as described by Wang et al. (98) with minor modifications. HSF grown to confluence in 100 mm culture dishes were cholesterol loaded with non-lipoprotein cholesterol, equilibrated, and then treated with 5 μM LXR Agonist (TO-901317) in DMEM/BSA for 24 h to upregulate ABCA1 expression. The cells were washed twice with PBS and then treated with DMEM/BSA or same media containing 10 μg/mL apoA-I, HDL, or tyrHDL in DMEM/BSA for 2 hrs at 37°C. Cells were then placed on ice for 15 minutes and washed three times with PBS. DSP (cross-linker agent) was dissolved immediately before use in dimethyl sulfoxide and diluted to 250 μM with PBS. 10 ml of DSP solution was added into each dish. Cells were then incubated at room temperature for 1 h, the medium was removed, and the cells were washed twice with iced-PBS. Cells were lysed at 4 °C with IP buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton X-100 (BDH Chemicals), and complete protease inhibitor (Roche Molecular Biochemicals). The cells were lysed by

aspiration with a fine-tipped needle, and the mixture was left to rotate at 4°C for 30 min. Cell lysates were centrifuged at 1,500 RPM for 10 min at 4°C to remove cellular debris. The supernatant was collected and incubated with Laemmli sample buffer in the absence or presence of β-mercaptoethanol before loading onto 4-20% gradient SDS-PAGE gels for electrophoresis followed by electrotransfer to a nitrocellulose sheet for autoradiography and Western analysis. ApoA-I cross-linked to ABCA1 was detected by blotting the membranes with rabbit polyclonal anti-human apoA-I antibody (1:10,000) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000). To detect ABCA1 alone, the membrane was stripped and reprobed with rabbit polyclonal antibody to ABCA1 (1:1000) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000).

2.2.10 Cell surface binding of intact and trypsinized HDL and tyrHDL

To assess cell surface binding of intact and trypsinized HDL and tyrHDL, HSF were grown to confluence on 35 mm wells in DMEM containing 10% FBS, loaded with non-lipoprotein cholesterol, equilibrated, and then rinsed three times with warm PBS/BSA. The cells were then chilled and left on ice for 5 min before incubation for 3 h on ice at 4°C in DMEM/BSA, or same media plus 0-20 μM based on phospholipid content of intact HDL or tyrHDL, or trypsinized HDL or tyrHDL. The cells were then washed three times with iced PBS/BSA and then incubated with DMEM/BSA containing 2 μg/mL ^{125}I-HDL for 1 h on ice. After this incubation, the cells were rinsed five times with iced PBS/BSA and twice with iced PBS. One mL of 0.1 N NaOH was added to each well, and the cells were left to shake for 1 h on a rotary shaker. Aliquots of 0.5 mL from each well were taken for ^{125}I counts and aliquots of 0.2 mL were taken for total cell protein determination by the Lowry assay (164) using BSA as standard.

2.2.11 2-Dimensional Gel Electrophoresis of HDL particles

HSF were grown to confluence on 35 mm wells in DMEM containing 10% FBS, then loaded with non-lipoprotein cholesterol and equilibrated. Cells were then treated with 10 μg/mL of apoA-I, HDL, or tyrHDL for 24 h. Cells were washed twice with PBS/BSA and then incubated with 1 mL of 10 μg/mL apoA-I for 24 h. To characterize

apoA-I-containing particles generated by HSF, fibroblast-conditioned media in the 35 mm dishes were centrifuged at 2000 rpm for 5 min at 4°C to pellet cells and the supernatant was concentrated 10-fold by ultrafiltration (Amicon Ultra-4, MWCO 10000, Millipore). Media samples were kept on ice and used the same day or frozen at -80°C. Plasma HDL particles were separated according to the method of Asztalos *et al.* as previously described (68). HDL particles in equivalent volumes of concentrated apoA-I-conditioned media were separated according to the method of Castro and Fielding (166) except that in the second dimension, voltage was increased from 100V for 19 h to 125V for 24 h to increase the separation of the α-migrating HDL species. Briefly, 20 µl samples were separated in the first dimension by 0.75% agarose gel in 50 mM barbital buffer, pH 8.6, at 200 V for 5.5 h at 5°C. Electrophoresis in the second dimension was performed with a 2-23% polyacrylamide concave gradient gel at 125 V for 24 h at 5°C in 0.025 M Tris, 0.192 M glycine buffer, pH 8.3. High molecular weight protein standards (7.1 to 17.0 nm, Amersham Pharmacia Biotech) were run on each gel. Following electrophoresis, samples were electrotransferred (30 V, 24 h, 4° C) onto nitrocellulose membranes (Trans-Blot, BioRad). To locate the standard proteins, the nitrocellulose membranes were stained with Ponceau S and the position of each protein marked. ApoA-I-containing particles were detected by blotting the membranes with rabbit polyclonal anti-human apoA-I antibody (1:10,000) and a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (1:10,000). Membranes were extensively rinsed with TBS/T before chemiluminescent detection using the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

CHAPTER 3: RESULTS

3.1 Tyrosylated HDL requires ABCA1 to function

Cell culture studies have suggested that tyrHDL is capable of mobilizing cellular cholesterol for efflux from a pool that would otherwise be esterified into CE by ACAT (155). TyrHDL was also shown to have no direct inhibitory effect on the activity of ACAT in whole cell homogenate assays (155), or in increasing the rate of CE hydrolysis by neutral cholesteryl ester hydrolase (156), suggesting the effect of tyrHDL is to enhance the removal of the regulatory substrate pool of FC from the cholesteryl ester cycle rather than directly inhibiting or enhancing the activity of the enzymes in this cycle. This same regulatory pool of cholesterol is thought to be acted upon by ABCA1, the cell membrane transporter that mediates cholesterol transport to extracellular acceptor molecules (58, 73-75). The proposed function of ABCA1 in mobilizing cholesterol for HDL formation and the demonstrated actions of tyrHDL show several similarities. Hence, we first wondered whether tyrHDL required ABCA1 to function. To determine whether this was true, we performed the cholesterol esterification assay – a measure of removal of the regulatory pool of cholesterol utilized by ACAT – in normal human skin fibroblasts (HSF), and in Tangier disease fibroblasts which lack functional ABCA1. In this assay, cholesterol-loaded cells were treated with albumin as control plus HDL or tyrHDL for 16 h to deplete the ACAT-substrate pool of cholesterol. Cells were then incubated with [^{14}C]oleate, and the amount of cholesteryl [^{14}C]oleate formed in 1 h was measured by extraction of cellular lipids and determination of radioactivity in cell CE. Consistent with previous observations (155), normal HSF treated with tyrHDL formed significantly less radiolabeled CE than cells treated with HDL (**Figure 3.1**), suggesting enhanced depletion of the regulatory pool of cholesterol by tyrHDL in these cells. However, the enhanced removal of ACAT-accessible cholesterol by tyrHDL was not seen in either of the Tangier disease cell lines when compared to HDL (**Figure 3.1**), indicating that tyrHDL, at minimum, requires the presence of functional ABCA1 to perform its enhanced effect. The residual depletion of ACAT-accessible cholesterol by HDL and tyrHDL in Tangier disease cells is likely due to depletion of PM cholesterol by non-ABCA1-dependent mechanisms, *e.g.*, passive diffusion, with depletion of some ACAT-accessible cholesterol to replenish PM cholesterol lost by these mechanisms.

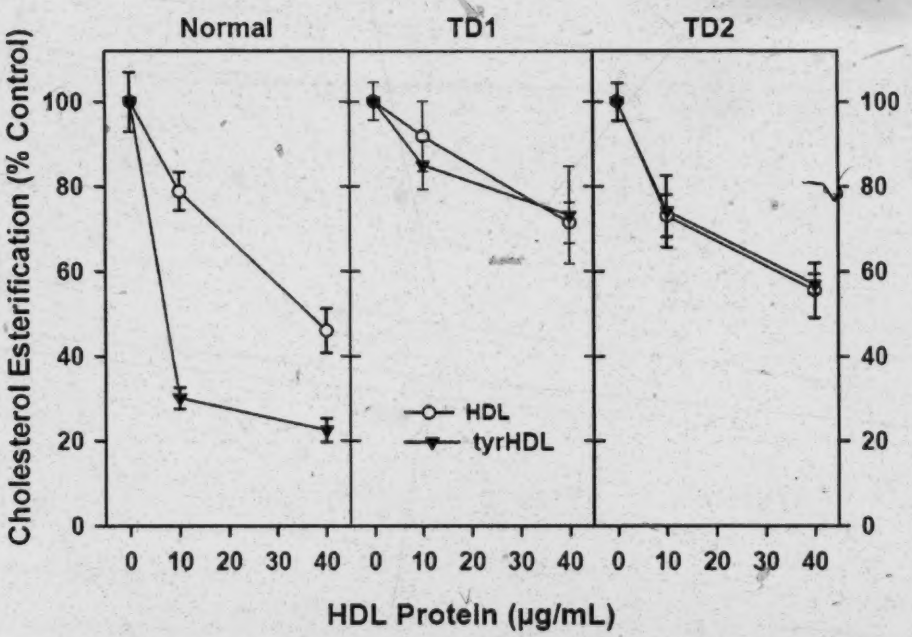

Figure 3.1: Absence of enhanced cholesterol mobilization by tyrHDL from Tangier disease fibroblasts. HSF from a normal donor and two unrelated Tangier disease subjects (TD 1 and TD2) were grown to confluence, loaded with non-lipoprotein cholesterol for 24 h, equilibrated for an additional 24 h, and incubated with medium containing 1 mg/mL albumin (control) plus the indicated concentrations of HDL or tyrHDL for 16 h. Cells were then washed and incubated with [^{14}C]oleate for 1 h, washed, and cellular lipids extracted for determination of incorporation of label into [^{14}C]cholesteryl ester, as a measure of the size of the cholesterol substrate pool for ACAT. Results are expressed as % of cholesterol esterification in non-HDL-containing medium. Avg±SD of quadruplicates, representative of 3 experiments.

3.2 Effect of tyrosylated HDL on ABCA1 mRNA and protein levels

The known ability of ABCA1 to mobilize ACAT-accessible cholesterol and the markedly enhanced ability of tyrHDL to induce removal of this ACAT substrate pool (**Figure 3.1**) suggested tyrHDL might be having a direct effect on ABCA1 expression and/or activity. Preliminary results from our laboratory suggested tyrHDL increases ABCA1 mRNA levels. To assess the effect of tyrHDL on ABCA1 expression, human aortic smooth muscle cells (HASMC) were cholesterol loaded with non-lipoprotein cholesterol or not, and then treated with albumin as a control in the absence or presence of 10 µg/mL HDL or tyrHDL for 2 or 16 h to assess early and later time points for the

exposure. Cholesterol loading increased ABCA1 mRNA levels, consistent with previous reports (**Figure 3.2**) (64, 76).

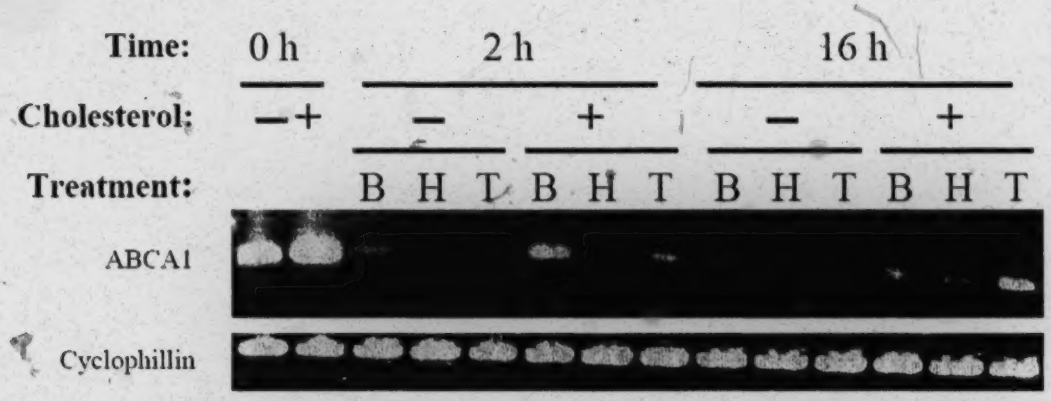

Figure 3.2: TyrHDL increases ABCA1 mRNA in human aortic smooth muscle cells. HASMC were grown to confluence and then cholesterol loaded or not with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h prior to treatment with albumin (B), or albumin plus 10 μg/mL HDL (H) or tyrHDL (T) for 2 or 16 h for determination of ABCA1 mRNA levels. Cyclophilin was used as loading control for reverse transcriptase-PCR. This is 1 experiment with no further repeats.

In all cases, cells treated with HDL resulted in lower ABCA1 mRNA levels when compared to incubations with albumin alone, consistent with the idea that as HDL passively removes cholesterol from intracellular stores, ABCA1 mRNA levels decrease (**Figure 3.2**) (64, 76). However, what was unexpected was that cells treated with tyrHDL for 2 h still retained high levels of ABCA1 mRNA when compared to cells treated with HDL (**Figure 3.2**), despite the apparent ability of tyrHDL to mobilize the regulatory pool of cholesterol utilized by ACAT. This was very apparent in the cholesterol-loaded cells treated with tyrHDL for 2 h. Similar results were seen when cells were treated with tyrHDL for 16 h, and again the effect was most pronounced in cholesterol-loaded cells (**Figure 3.2**). In all cases, ABCA1 mRNA levels remained high in cells incubated with albumin alone, as albumin is a relatively poor acceptor of cell cholesterol when compared to HDL.

Previous studies in our laboratory examining the effects of tyrHDL were carried out using HSF, which produced consistent and reliable results (155-157). Therefore, we also examined the effect of tyrHDL on ABCA1 mRNA levels in HSF. These

experiments were done using a single time point of 16 h. Unfortunately, experiments utilizing HSF produced variable results that did not agree to those seen in HASMC. In experiment 1, cholesterol loading increased ABCA1 mRNA levels in HSF; however, in both cholesterol loaded and non-cholesterol-loaded cells, tyrHDL was not able to increase ABCA1 mRNA levels when compared to cells treated with HDL (**Figure 3.3**).

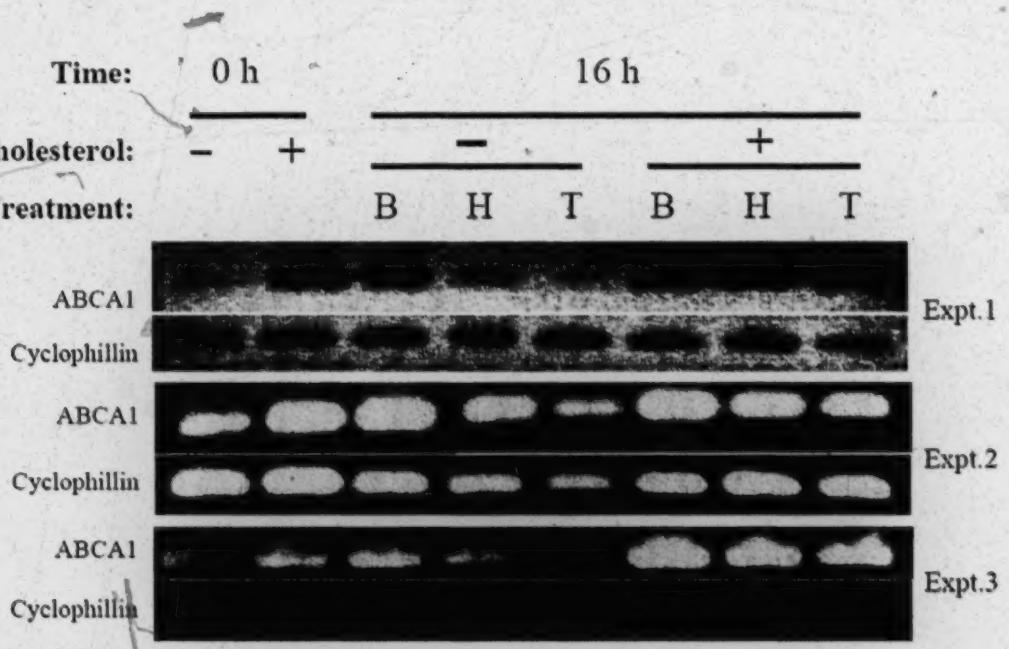

Figure 3.3: The effects of tyrHDL on ABCA1 mRNA in human skin fibroblasts. HSF were grown to confluence and then cholesterol loaded or not with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h prior to treatment with albumin (B), or albumin plus 10 μg/mL HDL (H) or tyrHDL (T) for 16 h for determination of ABCA1 mRNA levels. Cyclophilin was used as loading control for reverse transcriptase-PCR.

Variabilities were also seen in further experiments where there was an increase in ABCA1 mRNA in response to cholesterol loading, but no change in ABCA1 mRNA levels in HSF treated with albumin, HDL, or tyrHDL (**Figure 3.3**, experiment 2 and 3). Although our results with HASMC suggest a potential effect of tyrHDL on enhancing ABCA1 expression, our inability to reproduce this effect in HSF indicate further studies are required in HSF and other cell lines to know whether this is a general effect, or specific to HASMC.

Increased ABCA1 mRNA levels in HASMC treated with tyrHDL was associated with increased ABCA1 protein levels in these cells. Again, cholesterol-loading increases ABCA1 protein, and after 16 h of treatment with tyrHDL, both cholesterol-loaded and non-cholesterol-loaded HASMC showed increased ABCA1 protein when compared to cells treated with HDL, but this effect was more pronounced in cholesterol-loaded cells (**Figure 3.4**). In the cholesterol-loaded condition, HASMC treated with HDL had slightly higher ABCA1 protein than cells treated with albumin (**Figure 3.4**). This is different from that seen in ABCA1 mRNA when HASMC treated with HDL expressed less ABCA1 mRNA than cells treated with albumin after 16 h of treatment (**Figure 3.2**).

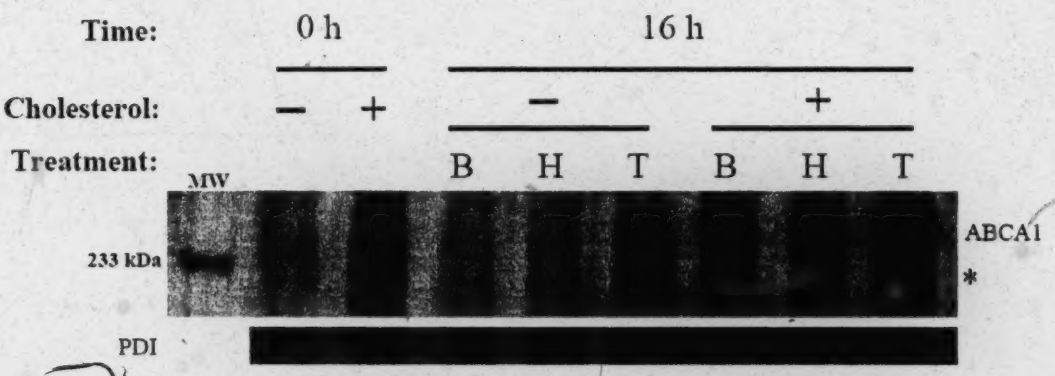

Figure 3.4: TyrHDL increases ABCA1 protein in human aortic smooth muscle cells. HASMC were grown to confluence and then cholesterol loaded or not with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h prior to treatment with albumin (B), or albumin plus 10 μg/mL HDL (H) or tyrHDL (T) for 16 h for determination of ABCA1 protein levels. PDI was used as loading control. * This band is likely non-specific.

Despite no definite increase in ABCA1 mRNA levels in HSF, our prior results with respect to the cholesterol esterification assay in response to tyrHDL suggested a likely effect of tyrHDL on ABCA1 activity in human fibroblasts. We therefore repeated our studies on the effects of tyrHDL on ABCA1 protein levels in HSF. Unlike HASMC, non-cholesterol-loaded HSF treated with tyrHDL showed no increase in ABCA1 protein compared to cells treated with HDL (**Figure 3.5**). However, similar to HASMC, cholesterol-loading increased basal ABCA1 protein levels in HSF, and in the cholesterol-loaded condition, treatment with tyrHDL yielded increased ABCA1 protein compared to

cells treated with HDL (**Figure 3.5**). Furthermore, cholesterol-loaded HSF treated with HDL expressed slightly higher levels of ABCA1 protein than those treated with albumin, which is consistent with our earlier findings in HASMC (**Figure 3.4**).

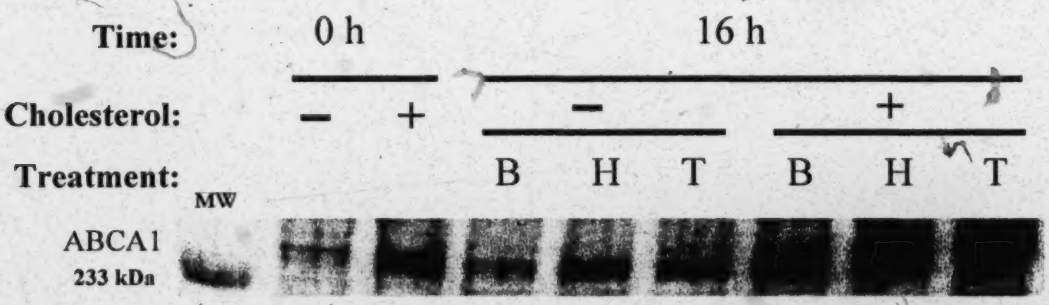

Figure 3.5: TyrHDL increases ABCA1 protein in human skin fibroblasts. HSF were grown to confluence and then cholesterol loaded or not with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h prior to treatment with albumin (B), or albumin plus 10 μg/mL HDL (H) or tyrHDL (T) for 16 h for determination of ABCA1 protein levels. Results shown are representative of two experiments with similar results.

Therefore, what has been consistent and reproducible is that the enhanced activity of tyrHDL on ABCA1 protein concentration can be demonstrated in HASMC and HSF, and this effect is more pronounced in the cholesterol-loaded condition, an environment that is representative of atherosclerotic lesions. It would seem that tyrHDL functions best in conditions of cholesterol loading; hence, all remaining experiments in the thesis were performed under conditions of excess cholesterol enrichment and using HSF unless otherwise stated.

3.3 Tyrosylated HDL requires LXR to function

The fact that tyrHDL was able to increase ABCA1 mRNA and protein in HASMC in non-cholesterol-loaded cells (**Figure 3.2 and 3.4**) had interesting potential implications. In cholesterol-depleted cells, the concentrations of oxysterols – metabolites of cholesterol and natural ligands of LXR – should be minimal. LXR is known to drive the transcription of the *ABCA1* gene (77, 78), however, our results would imply that tyrHDL might be functioning to increase ABCA1 expression and cholesterol mobilization in an LXR-independent manner. Earlier studies in our laboratory had

shown an ability of tyrHDL to further deplete ACAT-accessible cholesterol even in cells starved of cholesterol by growth to confluence in lipoprotein-deficient serum (Francis, G.A., unpublished data). This further suggested tyrHDL might be enhancing ABCA1 expression by an oxysterol- and therefore LXR-independent mechanism. To determine whether tyrHDL requires LXR to show its enhanced effect, we obtained mouse skin fibroblasts (MSF) deficient in both LXR α and β from Dr. David Mangelsdorf, University of Texas Southwestern, and compared these to mouse fibroblasts obtained from wild type mice of the same strain (C57BL/6) in their ability to release ACAT-accessible cholesterol to tyrHDL. As seen with HSF (**Figure 3.1**) (155), wild-type MSF treated with tyrHDL showed increased depletion of the ACAT substrate pool of cholesterol available for esterification when compared to MSF treated with HDL (**Figure 3.6A**). However, when MSF deficient in LXR α and β were treated with tyrHDL, there was less depletion of ACAT-accessible cholesterol than in cells treated with HDL (**Figure 3.6B**), suggesting that the enhanced ability of tyrHDL to deplete the ACAT-accessible pool of cholesterol is indeed dependent on the availability of LXR. The difference in the ability of HDL and tyrHDL to mobilize cholesterol for efflux from LXR α- and β-deficient cells can be explained by the fact that HDL is a slightly better acceptor of cholesterol efflux by passive diffusion than is tyrHDL (156). There is an enhanced ability of normal HDL to deplete the ACAT-accessible pool of cholesterol in the LXR double knockout MSF (**Figure 3.6B**) than is normally seen (**Figure 3.1 and 3.6A**). Cells from the adrenal gland of LXR double knockout mice have been shown to express residual ABCA1 protein (167). The phenomenon seen with the marked ability of native HDL to deplete the ACAT substrate pool of cholesterol in the LXR double knockout MSF may come from increased apoprotein-dependent efflux due to apoproteins dissociating from the HDL surface – other studies we've done using ultracentrifugation show apoproteins dissociate more readily off HDL than tyrHDL (Francis, G.A., unpublished data). The effect that was lost however, is the ability of tyrHDL to mobilize the ACAT-accessible pool of cholesterol in MSF lacking both LXR isoforms. This may perhaps be due to the inability of tyrHDL to induce ABCA1 expression or increase ABCA1 protein in those cells.

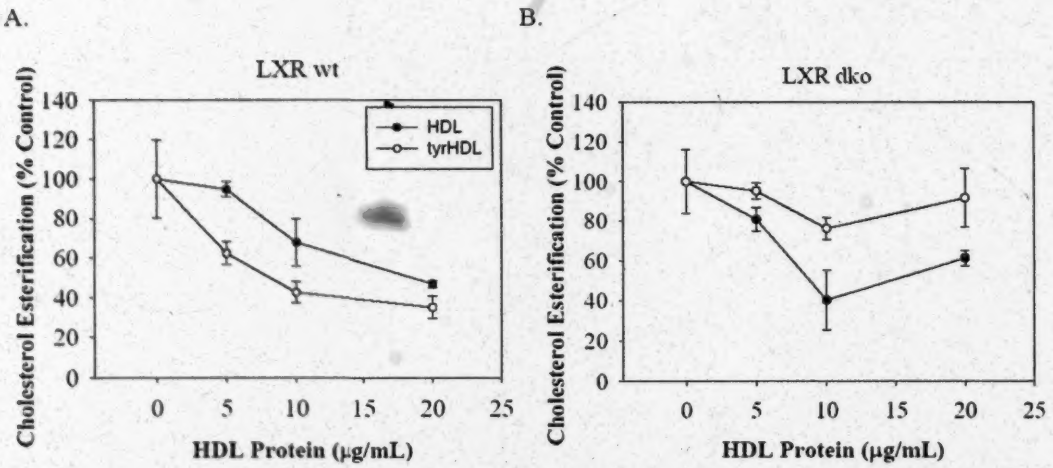

Figure 3.6: TyrHDL activity requires the presence of LXR. LXR wildtype and LXR double knockout MSF were loaded with non-lipoprotein cholesterol for 24 h, equilibrated for an additional 24 h, and incubated with medium containing 1 mg/mL albumin (control) plus the indicated concentrations of HDL or tyrHDL for 16 h. Cells were then washed and incubated with [^{14}C]oleate for 1 h, washed, and cellular lipids extracted for determination of incorporation of label into [^{14}C]cholesteryl ester, as a measure of the size of the cholesterol substrate pool for ACAT. Results are expressed as % of cholesterol esterification in non-HDL-containing medium. Avg±SD of triplicates, representative of 2 experiments with similar results.

3.4 Tyrosylated HDL stabilizes ABCA1 protein levels much like apoA-I

It has been reported that apoA-I is capable of protecting ABCA1 protein against calpain-mediated proteolysis, without affecting ABCA1 mRNA levels, thereby increasing ABCA1 protein (111, 112). HDL, on the other hand, does not exhibit this protective effect on stabilization of ABCA1 (111, 112). We had wondered whether tyrHDL was able to increase ABCA1 protein levels as much as apoA-I. To test whether tyrHDL can increase ABCA1 expression similar to apoA-I, normal HSF were cholesterol-loaded or not with non-lipoprotein cholesterol and then treated with either albumin alone or with addition of 10 μg/mL of apoA-I, HDL, or tyrHDL for 16 h, and crude membrane proteins extracted and probed for ABCA1 expression by Western blot. As a positive control, cholesterol-loaded HSF expressed greater ABCA1 protein as compared to their non-

cholesterol-loaded counterpart, while cells treated with apoA-I had increased ABCA1 protein expression compared to cells treated with albumin (**Figure 3.7**).

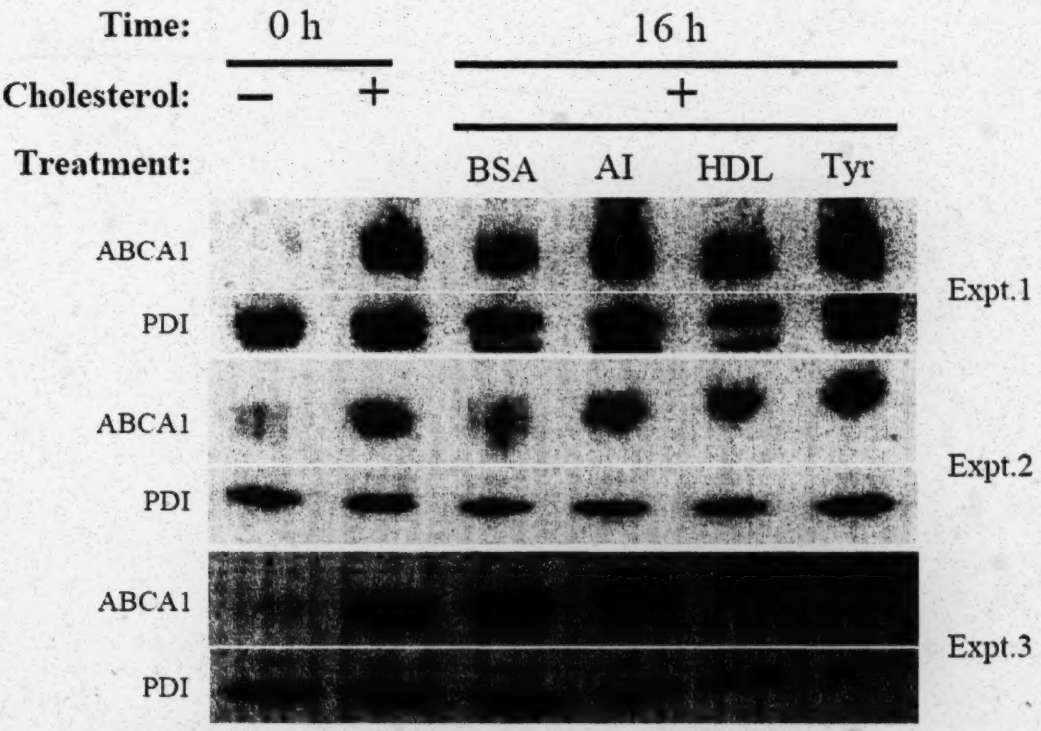

Figure 3.7: TyrHDL increases ABCA1 protein expression much like apoA-I in human skin fibroblasts. HSF were grown to confluence and then cholesterol loaded or not with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h prior to treatment with albumin (BSA), or albumin plus 10 μg/mL apoA-I (AI), HDL or tyrHDL (Tyr) for 16 h for determination of ABCA1 protein levels. PDI was used as loading control. Three independent experiments are shown with similar results.

Similarly, cells treated with tyrHDL expressed greater ABCA1 protein than cells treated with HDL (**Figure 3.7**), suggesting that tyrHDL can increase ABCA1 protein much like apoA-I.

3.5 Possible effects of tyrHDL on cell surface ABCA1 expression

ApoA-I has been reported to increase total and cell surface ABCA1 protein levels (113). To determine whether the increase in total ABCA1 protein in cells exposed to tyrHDL (**Figures 3.4, 3.5 and 3.7**) is also associated with increased cell surface ABCA1,

biotinylation experiments were performed. Cholesterol-loaded HSF were treated with either albumin alone or with addition of 10 μg/mL apoA-I, HDL, or tyrHDL for 16 h. Cells were then biotinylated to label cell surface proteins. Total and biotinylated cell surface ABCA1 were isolated and probed for ABCA1 levels by Western blot. The results I obtained between two individual experiments were inconsistent. Experiment 1 revealed that apoA-I is able to increase total ABCA1 relative to control, but there was no difference in the ability between HDL and tyrHDL to influence ABCA1 protein expression (**Figure 3.8**).

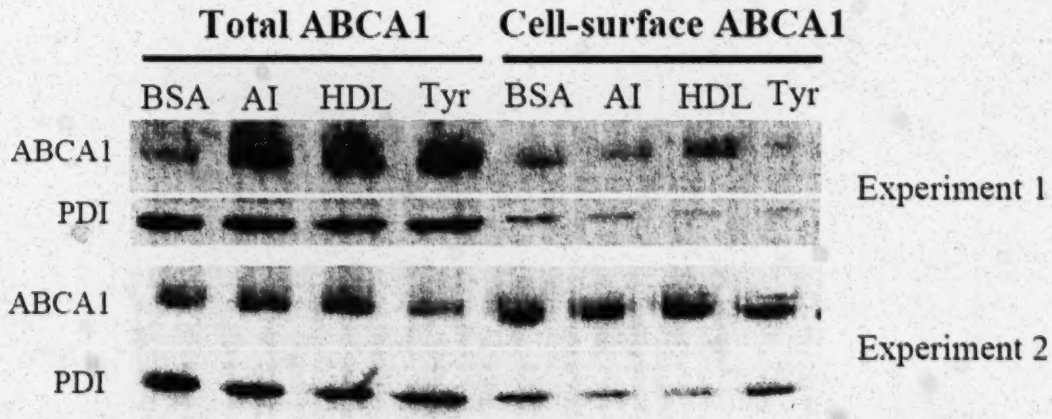

Figure 3.8: Total and cell surface expression of ABCA1 from human skin fibroblasts treated with tyrHDL. HSF were grown to confluence and then cholesterol loaded with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h prior to treatment with albumin (BSA), or albumin plus 10 μg/mL apoA-I (AI), HDL or tyrHDL (Tyr) for 16 h. Cells were then biotinylated and total and cell surface ABCA1 levels were determined by Western blotting. Two independent experiments are shown.

In fact, cells treated with apoA-I, HDL and tyrHDL expressed similar levels of total ABCA1, a finding that contradicts our previous results (**Figure 3.7**). Experiment 1 also showed that cells treated with apoA-I or tyrHDL expressed less cell surface ABCA1 than cells treated with albumin or HDL, respectively (**Figure 3.8**). This was unexpected because apoA-I was our positive control and should have caused an increase in cell surface ABCA1 (113). Experiment 2 revealed that there was not much difference in the ability of apoA-I, HDL or tyrHDL to influence total or cell surface ABCA1 expression relative to control. Interestingly, PDI, an endoplasmic reticulum protein was also isolated

in the fraction of cell surface proteins, suggesting contamination by cytoplasmic proteins, despite the fact that HSP90, a cytoplasmic protein, did not coelute with the cell surface fraction. In the absence of the expected increase in total and cell surface ABCA1 protein in response to apoA-I (113), our positive control, we cannot conclude from these experiments whether or not the increase in total cell ABCA1 seen in response to tyrHDL in **Figure 3.7**, where apoA-I did have the expected effect on total cell ABCA1, also results in increased cell surface ABCA1. Further repeats of this experiment where the expected results with apoA-I are also seen are required to resolve this question.

3.6 Tyrosylated HDL directly cross-links to ABCA1

Numerous studies using the chemical cross-linker dithiobis(succinimidyl propionate) (DSP) have suggested that apoA-I can directly bind to ABCA1, whereas HDL does not (93, 98, 106). We were curious to determine whether the enhanced ability of tyrHDL to redirect cellular cholesterol for efflux correlates with its ability to directly interact with ABCA1. We had theorized that the activity of tyrHDL did not depend on its ability to directly bind to ABCA1, because native HDL was shown not to directly cross-link to ABCA1 (98). In addition, our lab had previously found that apoproteins on the surface of tyrHDL dissociate less readily from the particle surface than from HDL when exposed to high speed ultracentrifugation (data not shown), suggesting a lesser ability of tyrHDL to release free apoproteins for interaction with ABCA1. To test this hypothesis, cholesterol-loaded HSF were treated with 5 μM of the LXR agonist TO-901317 to upregulate cell surface ABCA1 protein expression. The cells were then incubated with either albumin alone or with addition of 10 μg/mL of apoA-I, HDL, or tyrHDL for 2 h, the cells extensively washed, and then incubated with DSP for an additional 1 h. Cells were then washed and whole cell lysate was isolated and proteins were separated by a 4-20% SDS-PAGE and the presence of an apoA-I/ABCA1 cross-link was assessed by Western blot with an anti-apoA-I antibody, the membrane stripped, and then reprobed with an anti-ABCA1 antibody on the same nitrocellulose membrane. Any bands that overlap between the two blots at the expected molecular weight would suggest an apoA-I/ABCA1 cross-link. As expected, apoA-I was able to cross-link to ABCA1 with an estimated molecular weight of 250-280 kDa (**Figure 3.9A**).

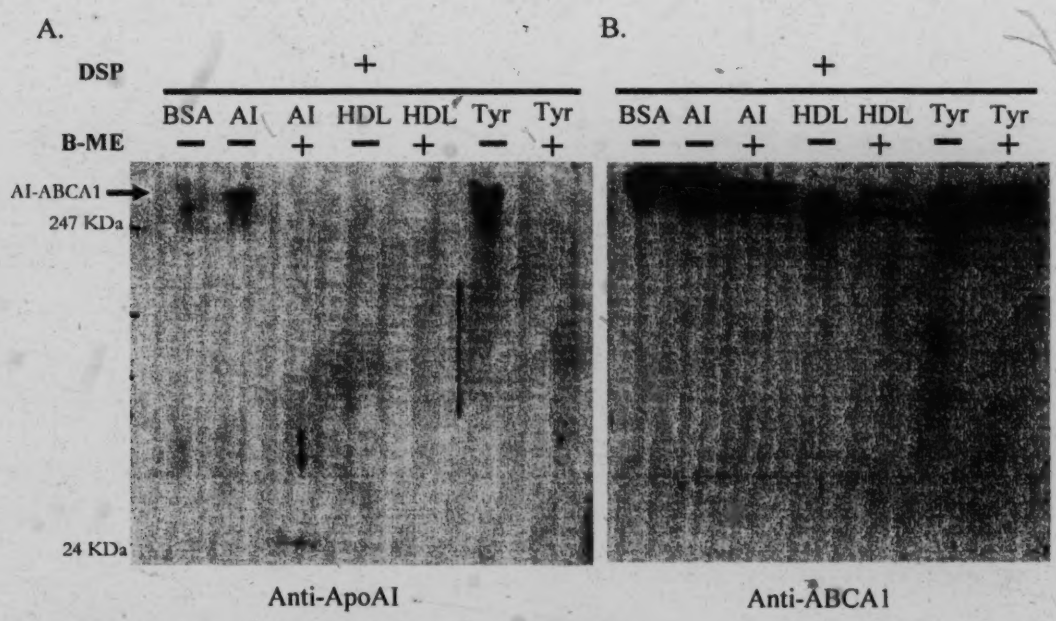

Figure 3.9: TyrHDL cross-links with ABCA1. HSF were grown to confluence and then loaded with non-lipoprotein cholesterol for 24 h and equilibrated with 5 μM of the LXR agonist TO-901317 for an additional 24 h to upregulate ABCA1 protein expression. The cells were then treated with albumin (BSA), or albumin plus 10 μg/mL apoA-I (AI), HDL or tyrHDL (Tyr) for 2 h, cells washed, and then incubated with 250 μM DSP for 1 h. Whole cell lysates were isolated, ran on a 4-20% gradient SDS-PAGE, and then probed for the presence of (A) apoA-I and (B) ABCA1 by Western blot. 5% β-mercaptoethanol (β-ME) was used to cleave any apoA-I-ABCA1 cross-links. AI-ABCA1 indicates an apoA-I/ABCA1 cross-link. Results are representative of 3 separate experiments with similar results.

In the presence of β-mercaptoethanol, a reducing agent capable of cleaving the cross-link, free apoA-I was released, which migrated to its expected molecular weight of 28 kDa (**Figure 3.9A**). HDL failed to cross-link to ABCA1 (98) as expected (**Figure 3.9A**). Surprisingly, tyrHDL, like apoA-I, was able to cross-link to ABCA1 and addition of β-mercaptoethanol resulted in a faint banding pattern of tyrHDL protein bands (**Figure 3.9A**). Further repeats of this experiment confirmed the binding of tyrHDL to ABCA1. However, additional experiments are required to demonstrate more clearly the appearance of tyrHDL protein bands upon dissociation of tyrHDL from ABCA1. **Figure 3.9B** illustrates that ABCA1 is present in all conditions, which was expected. The

appearance of the ABCA1 protein band changes upon addition of β-mercaptoethanol, for reasons that are unknown.

3.7 Tyrosylated HDL depletes cellular cholesterol much like apoA-I

Since tyrHDL has an enhanced ability to mobilize cholesterol for efflux (156), we wanted to assess whether tyrHDL can stimulate the formation of HDL particles from fibroblasts as determined by 2-dimensional gel electrophoresis. To evaluate the ability of tyrHDL to stimulate HDL particle formation, cholesterol-loaded HSF were pretreated with either albumin alone or with addition of 10 μg/mL apoA-I, HDL, or tyrHDL for 24 h. The ability of this pretreatment to mobilize cholesterol to the PM for efflux to apoA-I was then assessed by washing the cells and incubating with 10 μg/mL of apoA-I for an additional 24 h to remove the cholesterol that was previously mobilized for efflux to generate HDL particles. The media were then collected, concentrated, and the types of HDL particles formed assessed by 2-dimensional gel electrophoresis and Western blot analysis. Fibroblasts pretreated with apoA-I generated less α-migrating HDL particles than cells pretreated with albumin (**Figure 3.10**), which was expected as the initial pretreatment with apoA-I would have depleted some of the cellular lipids that would have subsequently been removed by the chase apoA-I to form HDL particles. Fibroblasts pretreated with tyrHDL showed a similar pattern of α-HDL particle formation as cells pretreated with apoA-I, but less α-HDL than cells pretreated with albumin alone or HDL (**Figure 3.10**). This suggested an increased removal of cellular lipids during the initial incubation with tyrHDL, as seen with apoA-I. In addition, there was the appearance of a pre-β HDL species in cells pretreated with tyrHDL and subsequently incubated with apoA-I, suggesting a possible increase in formation of these particles in response to tyrHDL.

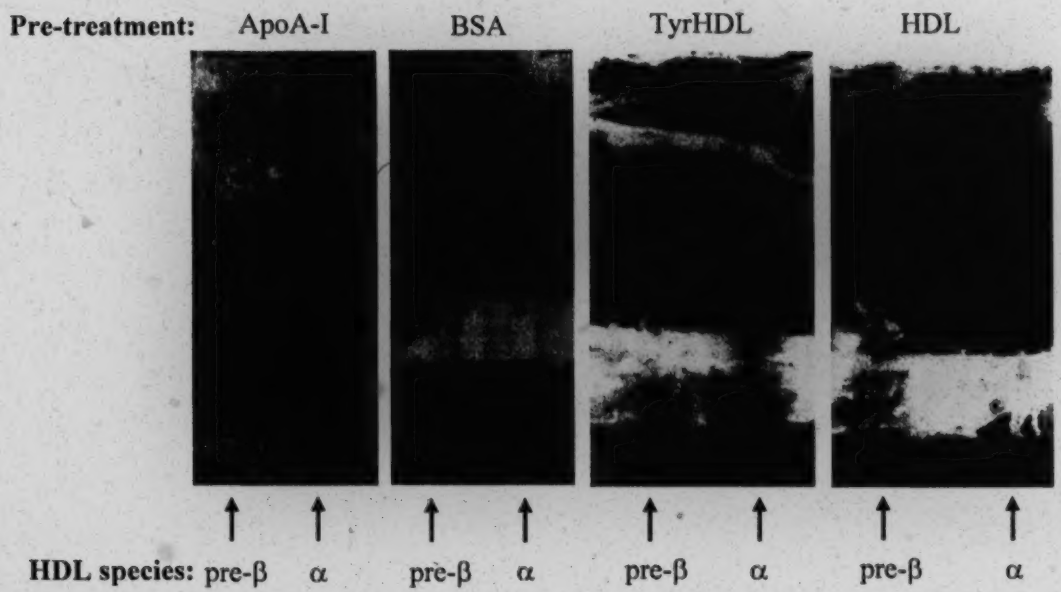

Figure 3.10: Formation of HDL particle species following pretreatment with tyrHDL. HSF were grown to confluence and then loaded with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h. The cells were then pretreated with albumin (BSA), or albumin plus 10 μg/mL apoA-I, HDL or tyrHDL for 24 h, cells washed, and then chased with 10 μg/mL free apoA-I for 24 h. The media were then collected, concentrated, and then analyzed for HDL particle species by 2-dimensional gel electrophoresis. Results are representative of 2 separate experiments with similar results.

However, this band may simply be free apoA-I, which migrates at pre-β mobility. These results suggest that tyrHDL is able to mobilize and stimulate the active efflux of cholesterol from cells much like apoA-I. Further repeats of this experiment are required to confirm these results.

3.8 Further work done in our laboratory

3.8.1 Protein fragments of tyrHDL can effectively mobilize cholesterol for efflux

Small peptide fragments modeled on the secondary structure of apoA-I have been shown to effectively reduce atherosclerosis in animal models (52, 53). Administration of tyrHDL into apoE-deficient mice resulted in elevations of endogenous HDL while protecting them against atherosclerosis (158). Consequently, we had wondered whether proteolytic fragments of tyrHDL would have similar activity as intact tyrHDL.

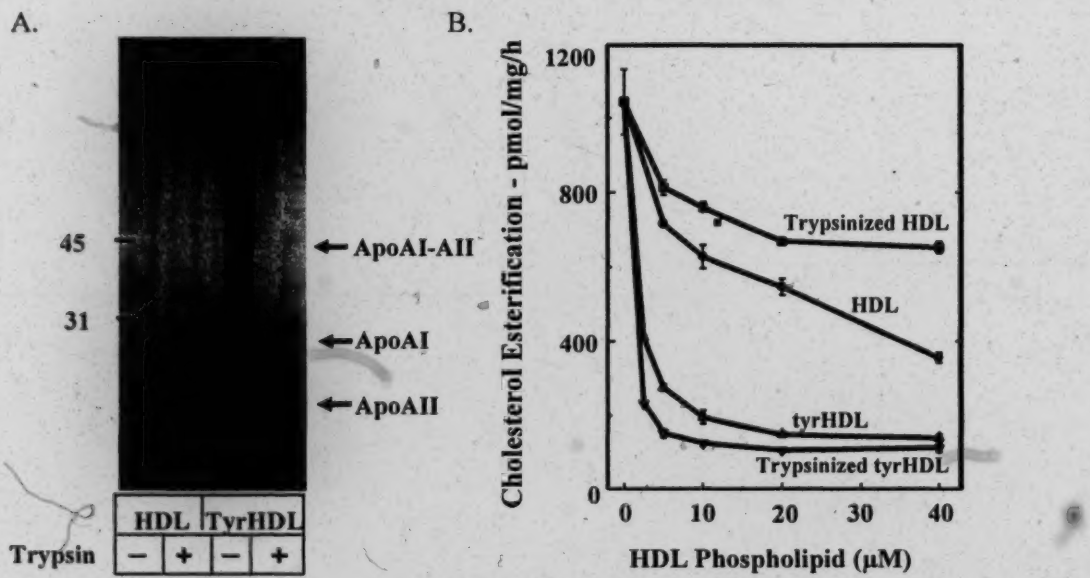

Figure 3.11: Full retention of cholesterol removal capacity by trypsinized tyrHDL. (A) HDL or tyrHDL were incubated in the absence or presence of trypsin for 30 min prior to analysis by SDS-PAGE gel with silver stain. (B) HSF were grown to confluence, loaded with non-lipoprotein cholesterol for 24 h, equilibrated for an additional 24 h, and incubated with medium containing 1 mg/mL albumin (control) plus the indicated concentrations of intact HDL, tyrHDL or trypsinized HDL or tyrHDL for 16 h. Cells were then washed and incubated with [^{14}C]oleate for 1 h, washed, and cellular lipids extracted for determination of incorporation of label into [^{14}C]cholesteryl ester, as a measure of the size of the cholesterol substrate pool for ACAT. Results are relative to cholesterol esterification in non-HDL-containing medium. Avg±SD of triplicates, representative of 4 experiments. Francis, G.A., unpublished data.

To investigate this idea, HDL and tyrHDL were subjected to a brief period of trypsinization to partially digest surface apoproteins, and the ability of the re-isolated digested and undigested particles to deplete ACAT-accessible cholesterol were

determined. Trypsinization of control HDL or tyrHDL resulted in the elimination of any intact apoprotein monomer and oligomers as well as apoAI-AII heterodimer bands (**Figure 3.11A**). It was discovered that trypsinized HDL mobilizes cholesterol less effectively than intact HDL, however trypsinized tyrHDL lost none of its ability to promote the removal of ACAT-accessible cholesterol when compared to intact tyrHDL (**Figure 3.11B**). This suggests that the tryptic fragments of tyrHDL proteins retain the same ability to mobilize cellular cholesterol as intact apoAI-AII heterodimers.

3.8.2 Trypsinized tyrHDL do not bind to fibroblasts

Since trypsin treatment of tyrHDL does not affect the ability of tyrHDL to induce cholesterol mobilization from cells, we tested whether trypsin-treated tyrHDL retains its ability to bind to cells as effectively as intact tyrHDL. Cholesterol-loaded fibroblasts were incubated with HDL, tyrHDL, or trypsinized HDL and tyrHDL at 4°C for 3 h, washed, and then incubated with ^{125}I-HDL for 1 h at 4°C to assess competition for binding of unlabeled lipoproteins with ^{125}I-HDL. We showed that intact tyrHDL competes less well than intact HDL in total cell binding assays, and that both trypsinized HDL and trypsinized tyrHDL lose their ability to bind to cells (**Figure 3.12**). The ability of trypsinized tyrHDL to promote depletion of the ACAT substrate pool similar to intact tyrHDL (**Figure 3.11B**) combined with the binding data suggests the enhanced effect of tyrHDL is not dependent on a strong binding interaction with ABCA1 or other potential HDL apoprotein binding sites on the surface of cholesterol-loaded fibroblasts.

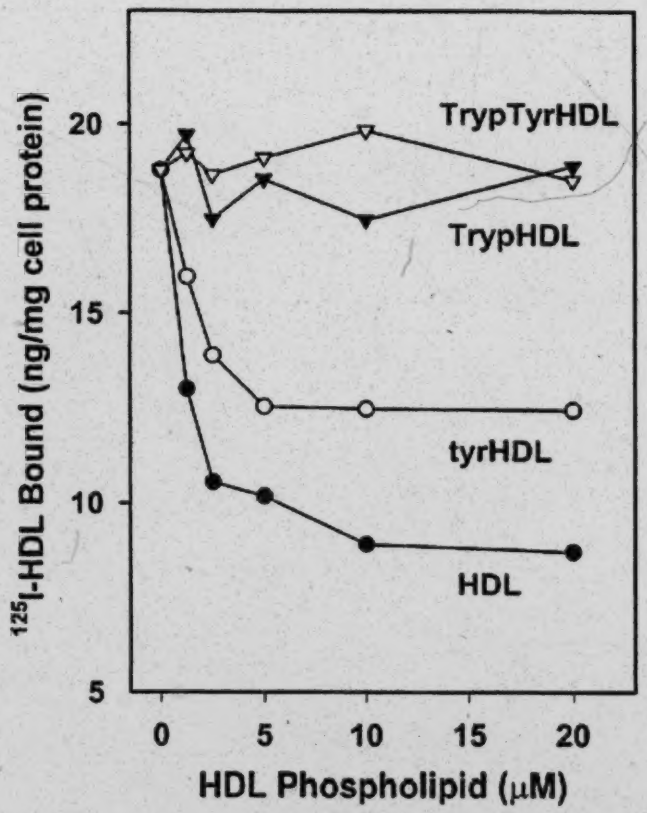

Figure 3.12: Binding of intact and trypsinized tyrHDL to human skin fibroblasts. HSF were grown to confluence and then loaded with non-lipoprotein cholesterol for 24 h and equilibrated for an additional 24 h. The cells were then incubated with HDL, tyrHDL, trypsinized HDL, or trypsinized tyrHDL at 0°C for 3 h, washed, and incubated with ^{125}I-HDL for 1 h at 0°C to assess competition for binding of unlabeled lipoproteins with ^{125}I-HDL. Avg. of duplicates, representative of 3 experiments. Francis, G.A., unpublished data.

CHAPTER 4: Discussion and Future Directions

We had previously shown that tyrHDL has a markedly enhanced ability when compared to HDL to mobilize cellular pools of cholesterol for efflux that would otherwise be esterified for storage into CE (155, 156). This effect of tyrHDL occurs even before cholesterol is effluxed out of cells (156), suggesting that tyrHDL stimulates an active process that shuttles cholesterol from the ACAT-accessible pool to an efflux-accessible pool on the PM. Interestingly, ABCA1 – the membrane protein that facilitates the export of cellular cholesterol to apoproteins – has also been shown to act in a similar fashion (58, 73-75). Studies completed for this thesis suggest the actions of tyrHDL require the presence of ABCA1, that tyrHDL increases ABCA1 protein and possibly mRNA levels, that tyrHDL, like apoA-I, stabilizes ABCA1 protein levels, and that tyrHDL, also like apoA-I but unlike HDL, is capable of being bound to ABCA1 by a cross-linking agent.

We wanted to determine whether tyrHDL required ABCA1 to function. Evidence for the requirement of functional ABCA1 for the action of tyrHDL was provided by studies showing a lack of enhancement of cholesterol mobilization by tyrHDL when compared to HDL in two fibroblast cell lines obtained from Tangier disease patients (**Figure 3.1**). The low level of residual cholesterol mobilization from Tangier disease cells can be explained by non-ABCA1-dependent efflux to HDL and tyrHDL from these cells (58).

Further studies suggested that cultured HASMC treated with tyrHDL showed persistence of ABCA1 mRNA expression (**Figure 3.2**). These results appeared in cells loaded with excess cholesterol or not, and despite the fact that tyrHDL is much better than HDL at removing the pool of cholesterol that upregulates ABCA1 expression. The normal response to the removal of cellular cholesterol by HDL is a rapid decrease in ABCA1 mRNA (64, 76), however ABCA1 mRNA in HASMC persists even after 16 h of incubation with tyrHDL (**Figure 3.2**). ABCA1 mRNA levels correlated well with ABCA1 protein levels in HASMC. HASMC treated with tyrHDL also showed increased ABCA1 protein in both cholesterol-loaded and non-cholesterol-loaded cells when compared to HDL (**Figure 3.4**). This is consistent with the idea that the enhanced activity of tyrHDL to mobilize cholesterol for efflux is due to an increased expression of ABCA1, the membrane transporter responsible for cholesterol efflux. As far as we know,

this is the first report of a modified HDL particle that is capable of increasing ABCA1 mRNA AND protein expression. In the cholesterol-loaded condition, HASMC treated with HDL had slightly higher ABCA1 protein than cells treated with albumin (**Figure 3.4**). Free apoA-I has been shown to prevent calpain-mediated proteolysis of ABCA1 without affecting ABCA1 mRNA levels, thereby increasing total ABCA1 protein (111, 112). Perhaps cells treated with HDL express slightly higher levels of ABCA1 protein than cells treated with albumin because some dissociation of apoA-I from the HDL particles can stabilize ABCA1 from calpain degradation. In the future, it would be of interest to determine whether there is a time and concentration-dependent effect of tyrHDL on ABCA1 expression in HASMC. However, we were not able to reproduce an increase in ABCA1 mRNA levels in HSF treated with tyrHDL when compared to HDL (**Figure 3.3**). Because the ability of tyrHDL to retain ABCA1 mRNA expression seen in HASMC was not reproducible in HSF, it suggests that this effect of tyrHDL may only be specific to HASMC. Further experiments to assess whether this effect can be generalized to other cell types including HSF will need to be performed.

Similar to HASMC, however, ABCA1 protein levels were increased in HSF treated with tyrHDL (**Figure 3.5**). In the cholesterol-loaded condition, tyrHDL was able to increase ABCA1 protein in HSF when compared to cells treated with HDL alone (**Figure 3.5**). However, this effect did not occur in non-cholesterol loaded HSF, but was observed in HASMC in non-cholesterol loaded cells (**Figure 3.4 vs Figure 3.5**). Therefore, what seems to be consistent and reproducible between the two cell lines is the ability of tyrHDL to increase ABCA1 protein in cholesterol-loaded cells, suggesting that tyrHDL functions best under conditions of excess cellular cholesterol. This is desirable because cells in atherosclerotic lesions are typically enriched with cholesterol.

ApoA-I has been reported to have no effect on ABCA1 mRNA levels but can protect ABCA1 against calpain proteolysis, thereby retaining higher ABCA1 protein levels compared with cells treated with albumin or HDL (111, 112). We also found that tyrHDL was able to retain ABCA1 protein levels much like apoA-I (**Figure 3.7**), suggesting that tyrHDL may also increase ABCA1 protein by inhibiting the degradation of ABCA1 by the calpain protease. It has been shown that an ABCA1 mutant lacking a PEST sequence is resistant to calpain-mediated degradation (112, 113). Experiments to

determine whether tyrHDL can protect ABCA1 from calpain proteolysis would entail transfecting human embryonic kidney cells with wildtype ABCA1 or ABCA1 lacking the PEST sequence, and treating these cells with tyrHDL. We would expect tyrHDL to stabilize ABCA1 from calpain degradation in cells transfected with wildtype ABCA1, but should have no effect on the mutant ABCA1. Another experiment would be to test the susceptibility of those transfected cells to increasing concentrations of exogenous calpain after pretreatment with tyrHDL. Again, tyrHDL should stabilize the wildtype ABCA1 from calpain degradation.

Studies have shown that apoA-I can increase total and cell surface ABCA1 protein expression (113), thereby enhancing ABCA1-mediated cholesterol efflux. Since tyrHDL is able to increase total ABCA1 protein, we had wanted to determine whether it can also increase cell surface ABCA1 expression. My experiments examining the effect of tyrHDL on cell surface ABCA1 levels yielded inconsistent data. The first experiment revealed that HSF treated with HDL (our negative control) expressed similar or even slightly higher levels of total and cell surface ABCA1 than cells treated with apoA-I [our positive control, (113)] and tyrHDL (**Figure 3.8**). Experiment 2 showed that there was not much difference in the ability of apoA-I, HDL, or tyrHDL to influence total or cell surface ABCA1 protein when compared to albumin treatment alone (**Figure 3.8**). These results are inconsistent with apoA-I [(113) and **Figure 3.7**] and tyrHDL (**Figure 3.4, 3.5 and 3.7**) both increasing total ABCA1 protein in HASMC and HSF when compared to cells treated with albumin or HDL. Since our positive and negative controls did not work, it can be assumed that the results from these experiments are invalid and that repeat experiments where our controls show the expected results are necessary to answer whether tyrHDL can increase cell surface expression of ABCA1. Furthermore, the presence of PDI (an endoplasmic reticulum protein) in the cell surface fraction indicates contamination of this fraction by intracellular proteins. These inconsistent findings may be explained by the methodology. The manufacturer of the biotinylation kit required the use of 2.5-3.5 mg of total cell protein, whereas we were only able to use 0.5-0.8 mg total due to the limitations of the cell type that were utilized. Other studies assessing the expression of cell surface ABCA1 were done by biotinylating all cell surface proteins, isolating biotinylated ABCA1 by immunoprecipitation with ABCA1 antibody, followed

by SDS-PAGE and detection of biotinylated ABCA1 by streptavidin and total ABCA1 with ABCA1 antibody. Perhaps the use of this method in our studies may generate data that are more consistent to our findings and those of other groups.

We have demonstrated that tyrHDL is capable of depleting cellular pools of cholesterol even in non-cholesterol-loaded cells – a condition whereby the concentrations of oxysterols would be minimal. In addition, we have found the enhanced activity of tyrHDL is due to its protein rather than lipid components (157), suggesting the action of tyrHDL is not due to it delivering oxysterols or other oxidized lipids to cells. In combination, these results suggest tyrHDL is enhancing ABCA1 activity by an oxysterol-, and therefore possibly LXR-, independent mechanism. However, experiments using LXR α- and β-deficient mouse fibroblasts suggest otherwise. When these fibroblasts were treated with tyrHDL and assessed for their ability to mobilize ACAT-accessible cholesterol, this effect was less than in cells treated with HDL (**Figure 3.6B**). However, tyrHDL was more effective than HDL in normal MSF (**Figure 3.6A**). This indicates that tyrHDL indeed requires LXR for its enhanced activity. HDL is a slightly better acceptor of cholesterol efflux by passive diffusion than is tyrHDL (156), which likely explains why HDL was better than tyrHDL at mobilizing ACAT-accessible cholesterol from LXR α- and β-deficient fibroblasts (**Figure 3.6B**). As well, the marked ability of HDL to deplete the ACAT accessible pool of cholesterol in mouse fibroblasts lacking both LXR isoforms may be due to the dissociation of some apoproteins from the HDL particles, which can then act as cholesterol acceptors. Perhaps tyrHDL was not able to efficiently mobilize cholesterol for efflux in LXR double knockout mouse fibroblasts because tyrHDL was not able to upregulate ABCA1 expression in those cells. An appealing future study would be to examine the effects of tyrHDL on ABCA mRNA and protein levels in the LXR α- and β-deficient fibroblasts. We would expect that tyrHDL would not increase ABCA1 expression in those fibroblasts. We do not believe that tyrHDL has an effect on LXR activity or expression, but rather only a requirement for LXR. To confirm this, we could examine the levels of LXR mRNA by Northern blot analysis or real-time PCR upon treatment of normal fibroblasts with tyrHDL. It could be that tyrHDL requires the function of LXR downstream from its role as a regulator of transcription. Since the active component of tyrHDL is the apoprotein fraction, and not

the oxidized lipids (157), it is unlikely that tyrHDL is delivering oxysterols to cells for the upregulation of ABCA1 transcription by LXR. Perhaps LXR is regulating the transcription of a gene that encodes a partner protein required for ABCA1 to function. However, no ABCA1-partner protein that is regulated at the transcriptional level by LXR has yet been identified; this theory is purely speculative at this time.

The ability of tyrHDL to directly interact with ABCA1 was assessed with cross-linking studies, much like those of apoA-I with ABCA1. We confirmed that HDL is not capable of cross-linking to ABCA1 whereas free apoA-I is capable of doing so (**Figure 3.9**), consistent with another report (98), and that intact tyrHDL is also able to cross-link to ABCA1 at a distance of <1.2 nm. The ability of tyrHDL to cross-link to ABCA1 occurs despite the fact that intact tyrHDL is a somewhat less efficient competitor for binding to cells than is HDL (**Figure 3.12**). The interaction of apoA-I with cells and hence possibly with ABCA1 have been shown to elicit a signal transduction response that includes activation of protein kinase A (168), protein kinase C (169), and Janus kinase 2 (170) for apoA-I-mediated stimulation of cholesterol efflux (**Figure 4.1A**). Free apoproteins are thought to be natural ligands of ABCA1 (98). Thus it is likely that the apoproteins of tyrHDL, much like free apoA-I, can directly and physically bind ABCA1 in a manner that stabilizes ABCA1 from degradation by calpain protease (**Figure 3.7**), thereby enhancing cholesterol mobilization and efflux. TyrHDL apoproteins are likely less exchangeable than HDL apoproteins due to tethering of apoA-I by the more lipophilic apoA-II to the tyrHDL particle surface (157). Therefore, a model for the interaction of tyrHDL with ABCA1 can be envisioned whereby apoA-I is exposed and "extended" away from the tyrHDL particle, but still tethered to apoA-II that is sitting on the surface of the particle (**Figure 4.1C**). The "extended" apoA-I may then assume a more lipid-poor apoA-I conformation and directly interact with ABCA1. Normal HDL is unable to directly interact with ABCA1, possibly because its apoproteins assume an open conformation when lipidated on the HDL particle surface, rather than a free- or more lipid-poor conformation of apoA-I on the tyrHDL particle surface (**Figure 4.1B**). Whether or not this ability of tyrHDL to interact directly with ABCA1 is responsible for the enhanced stabilization of ABCA1 protein and increase in ABCA1 activity requires further investigation.

Trypsin fragments of tyrHDL proteins did not bind to the cell surface of cholesterol-loaded fibroblasts when compared to intact HDL (**Figure 3.12**), yet their ability to deplete the pool of cholesterol available for esterification – a process that is thought to be mediated by ABCA1 – was not lost (**Figure 3.11**). These results imply that trypsin fragments of tyrHDL are capable of enhancing ABCA1 activity, possibly through

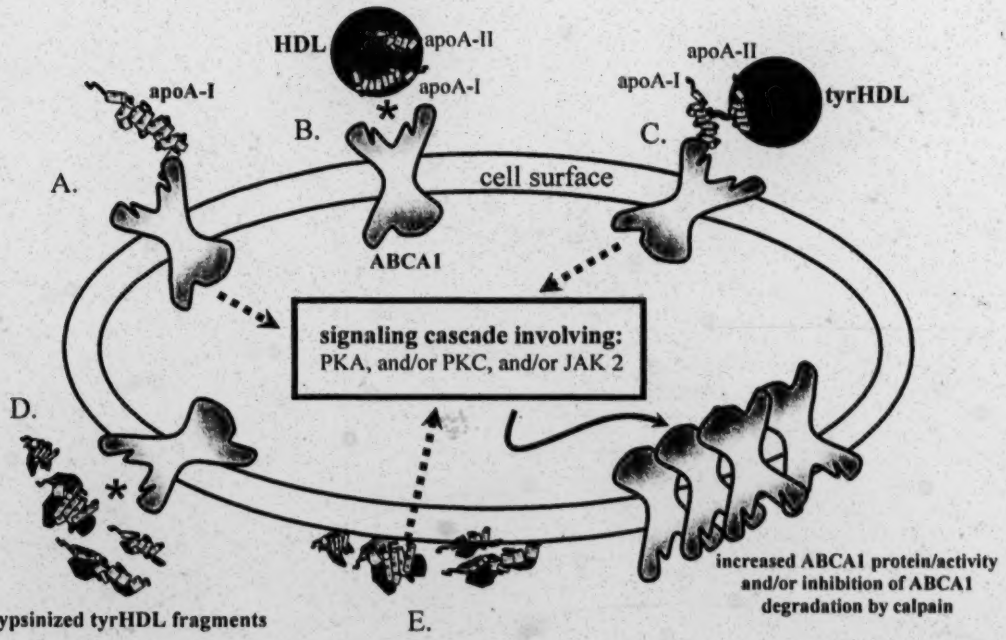

Figure 4.1. Models for the interaction of apoA-I, HDL, tyrHDL and trypsinized HDL with ABCA1. (A) Free apoA-I directly binds ABCA1 to elicit a signaling cascade that involves protein kinase A (PKA), and/or protein kinase C (PKC), and/or Janus kinase 2 (JAK 2) for the stabilization of ABCA1 from calpain-mediated degradation and/or an increase in the expression or activity of ABCA1 protein. (B) HDL does not directly bind to ABCA1, so that a signaling cascade is not induced. (C) The apoA-I of tyrHDL is complexed to apoA-II in such a way that apoA-I is extended from the tyrHDL surface where it may directly bind ABCA1 to elicit a signaling cascade much like free apoA-I. (D) Fragments of trypsinized tyrHDL may not directly bind with ABCA1. (E) Fragments of trypsinized tyrHDL may not have to directly bind to ABCA1 to elicit a signaling cascade for the stabilization of ABCA1 and/or increasing ABCA1 protein or activity. * Denotes inability to cross-link to ABCA1.

inhibition of calpain-dependent proteolysis, by a mechanism that does not require a direct ABCA1-apoprotein binding interaction (**Figure 4.1D and E**). They also suggest the action of intact tyrHDL in enhancing ABCA1 activity is not dependent on a direct

tyrHDL-ABCA1 interaction. It has been shown that binding of apoA-I to the cell surface activates a signal cascade involving many kinases (168-170). Interestingly, Tang and colleagues demonstrated that synthetic amphipathic helical apoprotein mimetic peptides and apoA-I can stabilize ABCA1 in the absence of an active Janus kinase 2, even though Janus kinase 2 is required for apoprotein cross-linking and cholesterol efflux (171). This would suggest that the stabilization of ABCA1 does not require the direct binding of amphipathic helical proteins such as apoA-I to ABCA1 (171). It could be that trypsinized fragments of tyrHDL apoproteins stimulate similar signal transduction pathways as intact apoA-I without having a direct relationship with ABCA1 (**Figure 4.1E**). Further studies are required to determine the effects of trypsinized tyrHDL on ABCA1 mRNA and protein levels.

We had known previously through cell culture experiments employing radiolabeled cholesterol that tyrHDL has an enhanced ability to mobilize cholesterol for efflux even before the initial removal of cholesterol from cells (156). Removal of this cholesterol was dependent on adequate concentrations of acceptor particles in the medium, those higher than 10 μg/mL, because there is little difference in the ability of tyrHDL or HDL to accept cholesterol by passive diffusion when concentrations of these lipoproteins are 10 μg/mL or less (156). It was therefore surprising to discover that when cholesterol-loaded HSF were initially treated with 10 μg/mL of HDL or tyrHDL and then chased with 10 μg/mL of apoA-I, the media from cells pre-treated with tyrHDL contained less α-migrating HDL particles than media from cells pre-treated with HDL (**Figure 3.10**). This indicated that some of the cellular cholesterol had already been mobilized and removed during the initial incubation with tyrHDL. A similar decrease in the amount of α-migrating HDL particles was also seen in cells pretreated with 10 μg/mL of free apoA-I (**Figure 3.10**). Since tyrHDL does not have an increased ability to mediate PL efflux when compared to HDL (156), the HDL formed during the chase period with apoA-I must be cholesterol enriched after preincubation with tyrHDL. This would imply that like apoA-I, tyrHDL has an enhanced ability to redistribute AND promote cholesterol efflux even at low concentrations (≤10 μg/mL). It was interesting to observe that in this cell culture system, HDL particles exhibiting α-electrophoretic mobility (α-migrating HDL particles) were formed in the absence of LCAT, which

normally esterifies free cholesterol on the surface of HDL particles into cholesterol esters that migrate to the center of the particle to form spherical HDL particles. These α-migrating HDL particles have also been reported by another group using similar methods (105). However, these α-migrating particles are likely discoidal rather than spherical, and the fact that they migrate as α particles may only reflect their PL content, since another study using similar methods and cell type have shown that these particles are rich in phosphatidylinositol that migrate with α-electrophoretic mobility (105). The reason for the discrepancy in the results with respect to cholesterol mobilization versus actual cholesterol efflux in the methods using radiolabeled cholesterol and the 2D-gel electrophoresis system is not known. Further experiments are required to confirm these results. It will also be interesting to examine the lipid composition of the HDL particles produced in the medium by fractionating and isolating the HDL particles through density gradient ultracentrifugation.

The ability of tyrHDL to increase ABCA1 expression and consequently RCT has therapeutic implications. We do not envision the administration of whole tyrHDL particles or to increase tyrosyl oxidation *in vivo* for the treatment of atherosclerosis, but rather to understand effects of tyrHDL to develop potential new treatments. The fact that trypsin fragments of tyrHDL have the same activity as intact tyrHDL to mobilize cholesterol for efflux suggests administration of peptide fragments or mimetics of tyrHDL is a promising therapeutic approach. The use of apoA-I mimetic peptides such as the D-4F peptide has been reported to reduce atherosclerosis in animal models; however the mechanism of action of these peptides is thought to be by inhibiting apoA-I oxidation and thereby reducing inflammation in atherosclerotic lesions (52, 53, 172). The use of peptide fragments of tyrHDL may be far better than the D-4F peptide in that tyrHDL fragments may directly influence ABCA1 expression and hence the RCT pathway.

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